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(54) MUTATED IMMUNOGLOBULIN-BINDING POLYPEPTIDES

(57) An Fc-binding polypeptide of improved alkali stability, comprising a mutant of an Fc-binding domain of Staphylococcus Protein A (SpA), as defined by SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO:3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:22, SEQ ID NO 51 or SEQ ID NO 52 wherein at least the asparagine or serine residue at the position corresponding to position 11 in SEQ ID NO:4-7 has been mutated to an amino acid selected from the group consisting of glutamic acid, lysine, tyrosine, threonine, phenylalanine, leucine, isoleucine, tryptophan, methionine, valine, alanine, histidine and arginine.

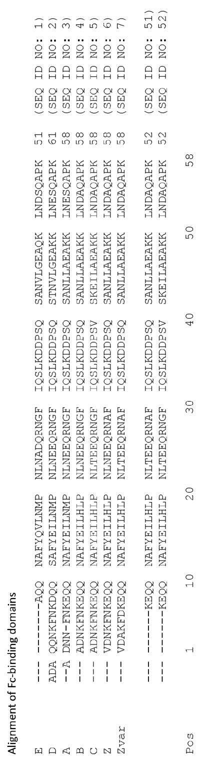

Fig. 1

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Description

Technical field of the invention

[0001] The present invention relates to the field of affinity chromatography, and more specifically to mutated immunoglobulin-binding domains of Protein A, which are useful in affinity chromatography of immunoglobulins. The invention also relates to multimers of the mutated domains and to separation matrices containing the mutated domains or multimers.

Background of the invention

[0002] Immunoglobulins represent the most prevalent biopharmaceutical products in either manufacture or development worldwide. The high commercial demand for and hence value of this particular therapeutic market has led to the emphasis being placed on pharmaceutical companies to maximize the productivity of their respective mAb manufacturing processes whilst controlling the associated costs.
[0003] Affinity chromatography is used in most cases, as one of the key steps in the purification of these immunoglobulin molecules, such as monoclonal or polyclonal antibodies. A particularly interesting class of affinity reagents is proteins capable of specific binding to invariable parts of an immunoglobulin molecule, such interaction being independent on the antigen-binding specificity of the antibody. Such reagents can be widely used for affinity chromatography recovery of immunoglobulins from different samples such as but not limited to serum or plasma preparations or cell culture derived feed stocks. An example of such a protein is staphylococcal protein A, containing domains capable of binding to the Fc and Fab portions of IgG immunoglobulins from different species. These domains are commonly denoted as the E-, D-, A-, B- and C-domains.
[0004] Staphylococcal protein A (SpA) based reagents have due to their high affinity and selectivity found a widespread use in the field of biotechnology, e.g. in affinity chromatography for capture and purification of antibodies as well as for detection or quantification. At present, SpA-based affinity medium probably is the most widely used affinity medium for isolation of monoclonal antibodies and their fragments from different samples including industrial cell culture supernatants. Accordingly, various matrices comprising protein A-ligands are commercially available, for example, in the form of native protein A (e.g. Protein A SEPHAROSE™, GE Healthcare, Uppsala, Sweden) and also comprised of recombinant protein A (e.g. rProtein A-SEPHAROSE™, GE Healthcare). More specifically, the genetic manipulation performed in the commercial recombinant protein A product is aimed at facilitating the attachment thereof to a support and at increasing the productivity of the ligand.
[0005] These applications, like other affinity chromatography applications, require comprehensive attention to definite removal of contaminants. Such contaminants can for example be non-eluted molecules adsorbed to the stationary phase or matrix in a chromatographic procedure, such as non-desired biomolecules or microorganisms, including for example proteins, carbohydrates, lipids, bacteria and viruses. The removal of such contaminants from the matrix is usually performed after a first elution of the desired product in order to regenerate the matrix before subsequent use. Such removal usually involves a procedure known as cleaning-in-place (CIP), wherein agents capable of eluting contaminants from the stationary phase are used. One such class of agents often used is alkaline solutions that are passed over said stationary phase. At present the most extensively used cleaning and sanitizing agent is NaOH, and the concentration thereof can range from 0.1 up to e.g. 1 M, depending on the degree and nature of contamination. This strategy is associated with exposing the matrix to solutions with pH-values above 13. For many affinity chromatography matrices containing proteinaceous affinity ligands such alkaline environment is a very harsh condition and consequently results in decreased capacities owing to instability of the ligand to the high pH involved.
[0006] An extensive research has therefore been focused on the development of engineered protein ligands that exhibit an improved capacity to withstand alkaline pH-values. For example, Gülich et al. (Susanne Gülich, Martin Linhult, Per-Ake Nygren, Mathias Uhlén, Sophia Hober, Journal of Biotechnology 80 (2000), 169-178) suggested protein engineering to improve the stability properties of a Streptococcal albumin-binding domain (ABD) in alkaline environments. Gülich et al. created a mutant of ABD, wherein all the four asparagine residues have been replaced by leucine (one residue), aspartate (two residues) and lysine (one residue). Further, Gülich et al. report that their mutant exhibits a target protein binding behavior similar to that of the native protein, and that affinity columns containing the engineered ligand show higher binding capacities after repeated exposure to alkaline conditions than columns prepared using the parental non-engineered ligand. Thus, it is concluded therein that all four asparagine residues can be replaced without any significant effect on structure and function.
[0007] Recent work shows that changes can also be made to protein A (SpA) to effect similar properties. US patent application publication US 2005/0143566, which is hereby incorporated by reference in its entirety, discloses that when at least one asparagine residue is mutated to an amino acid other than glutamine or aspartic acid, the mutation confers an increased chemical stability at pH-values of up to about 13-14 compared to the parental SpA, such as the B-domain of SpA, or Protein Z, a synthetic construct derived from the B-domain of SpA (US 5,143,844, incorporated by reference

in its entirety). The authors show that when these mutated proteins are used as affinity ligands, the separation media as expected can better withstand cleaning procedures using alkaline agents. Further mutations of protein A domains with the purpose of increasing the alkali stability have also been published in WO 2008/039141, JP 2006304633A, EP 1992692A1, EP 2202310A2, WO 2010/110288, WO 2012/086660, WO 2012/083425, WO 2012/087230 and WO 2014/146350, all of which are hereby incorporated by reference in their entireties. However, the currently available mutants are still sensitive to alkaline pH and the NaOH concentration during cleaning is usually limited to 0.1 M, which means that complete cleaning is difficult to achieve. Higher NaOH concentrations, which would improve the cleaning, lead to unacceptable capacity losses.

[0008] There is thus still a need in this field to obtain a separation matrix containing protein ligands having a further improved stability towards alkaline cleaning procedures.

Summary of the invention

[0009] One aspect of the invention is to provide a polypeptide with improved alkaline stability. This is achieved with a polypeptide as defined in claim 1.

[0010] One advantage is that the alkaline stability is improved over the parental polypeptides, with a maintained highly selective binding towards immunoglobulins and other Fc-containing proteins.

[0011] A second aspect of the invention is to provide a multimer with improved alkaline stability, comprising a plurality of polypeptides. This is achieved with a multimer as defined in the claims.

[0012] A third aspect of the invention is to provide a nucleic acid or a vector encoding a polypeptide or multimer with improved alkaline stability. This is achieved with a nucleic acid or vector as defined in the claims.

[0013] A fourth aspect of the invention is to provide an expression system capable of expressing a polypeptide or multimer with improved alkaline stability. This is achieved with an expression system as defined in the claims.

[0014] A fifth aspect of the invention is to provide a separation matrix capable of selectively binding immunoglobulins and other Fc-containing proteins and exhibiting an improved alkaline stability. This is achieved with a separation matrix as defined in the claims.

[0015] A sixth aspect of the invention is to provide an efficient and economical method of isolating an immunoglobulin or other Fc-containing protein. This is achieved with a method as defined in the claims.

[0016] Further suitable embodiments of the invention are described in the dependent claims.

Definitions

[0017] The terms "antibody" and "immunoglobulin" are used interchangeably herein, and are understood to include also fragments of antibodies, fusion proteins comprising antibodies or antibody fragments and conjugates comprising antibodies or antibody fragments.

[0018] The terms an "Fc-binding polypeptide" and "Fc-binding protein" mean a polypeptide or protein respectively, capable of binding to the crystallisable part (Fc) of an antibody and includes e.g. Protein A and Protein G, or any fragment or fusion protein thereof that has maintained said binding property.

[0019] The term "linker" herein means an element linking two polypeptide units, monomers or domains to each other in a multimer.

[0020] The term "spacer" herein means an element connecting a polypeptide or a polypeptide multimer to a support.

Brief description of figures

[0021]

Fig. 1 shows an alignment of the Fc-binding domains as defined by SEQ ID NO:1-7 and 51-52.

Fig. 2 shows results from Example 2 for the alkali stability of parental and mutated tetrameric Zvar (SEQ ID NO 7) polypeptide variants coupled to an SPR biosensor chip.

Fig. 3 shows results from Example 4 for the alkali stability (0.5 M NaOH) of parental and mutated tetrameric Zvar (SEQ ID NO 7) polypeptide variants coupled to agarose beads.

Fig. 4 shows results from Example 4 for the alkali stability (1.0 M NaOH) of parental and mutated tetrameric Zvar (SEQ ID NO 7) polypeptide variants coupled to agarose beads.

Detailed description of embodiments

[0022] In one aspect the present invention discloses an Fc-binding polypeptide, which comprises, or consists essentially of, a mutant of an Fc-binding domain of Staphylococcus Protein A (SpA), as defined by, or having at least 90%, at least 95% or at least 98% identity to, SEQ ID NO: 1 (E-domain), SEQ ID NO: 2 (D-domain), SEQ ID NO:3 (A-domain), SEQ ID NO:22 (variant A-domain), SEQ ID NO: 4 (B-domain), SEQ ID NO: 5 (C-domain), SEQ ID NO:6 (Protein Z), SEQ ID NO:7 (Zvar), SEQ ID NO 51 (Zvar without the linker region amino acids 1-6) or SEQ ID NO 52 (C-domain without the linker region amino acids 1-6) as illustrated in Fig. 1, wherein at least the asparagine (or serine, in the case of SEQ ID NO 2) residue at the position* corresponding to position 11 in SEQ ID NO:4-7 has been mutated to an amino acid selected from the group consisting of glutamic acid, lysine, tyrosine, threonine, phenylalanine, leucine, isoleucine, tryptophan, methionine, valine, alanine, histidine and arginine. Protein Z (SEQ ID NO:6) is a mutated B-domain as disclosed in US5143844, while SEQ ID NO 7 denotes a further mutated variant of Protein Z, here called Zvar, with the mutations N3A,N6D,N23T. SEQ ID NO:22 is a natural variant of the A-domain in Protein A from Staphylococcus aureus strain N315, having an A46S mutation, using the position terminology of Fig. 1. The mutation of N11 in these domains confers an improved alkali stability in comparison with the parental domain/polypeptide, without impairing the immunoglobulin-binding properties. Hence, the polypeptide can also be described as an Fc- or immunoglobulin-binding polypeptide, or alternatively as an Fc- or immunoglobulin-binding polypeptide unit.

[0023] *Throughout this description, the amino acid residue position numbering convention of Fig 1 is used, and the position numbers are designated as corresponding to those in SEQ ID NO 4-7.

[0024] In alternative language, the invention discloses an Fc-binding polypeptide which comprises a sequence as defined by, or having at least 90%, at least 95% or at least 98% identity to SEQ ID NO 53.

SEQ ID NO 53

KEX$_1$Q X$_2$AFYEILX$_3$LP NLTEEQRX$_4$X$_5$F IX$_6$X$_7$LKDX$_8$PSX$_9$ SX$_{10}$X$_{11}$X$_{12}$LAEAKX$_{13}$ X$_{14}$NDAQAPK

where individually of each other:

X_1=A or Q
X_2=E,K,Y,T,F,L,W,I,M,V,A,H or R
X_3=H or K
X_4=A or N
X_5=A or G
X_6=Q or E
X_7=S or K
X_8=E or D
X_9=Q or V
X_{10}=K,R or A
X_{11}=A,E or N
X_{12}=I or L
X_{13}=K or R
X_{14}=L or Y

[0025] The N11 (X_2) mutation (e.g. a N11E or N11K mutation) may be the only mutation or the polypeptide may also comprise further mutations, such as substitutions in at least one of the positions corresponding to positions 3, 6, 9, 10, 15, 18, 23, 28, 29, 32, 33, 36, 37, 40, 42, 43, 44, 47, 50, 51, 55 and 57 in SEQ ID NO:4-7. In one or more of these positions, the original amino acid residue may e.g. be substituted with an amino acid which is not asparagine, proline or cysteine. The original amino acid residue may e.g. be substituted with an alanine, a valine, a threonine, a serine, a lysine, a glutamic acid or an aspartic acid. Further, one or more amino acid residues may be deleted, e.g. from positions 1-6 and/or from positions 56-58.

[0026] In some embodiments, the amino acid residue at the position corresponding to position 9 in SEQ ID NO:4-7 (X_1) is an amino acid other than glutamine, asparagine, proline or cysteine, such as an alanine. The combination of the mutations at positions 9 and 11 provides particularly good alkali stability, as shown by the examples. In specific embodiments, in SEQ ID NO: 7 the amino acid residue at position 9 is an alanine and the amino acid residue at position 11 is a lysine or glutamic acid, such as a lysine. Mutations at position 9 are also discussed in copending application PCT/SE2014/050872, which is hereby incorporated by reference in its entirety.

[0027] In some embodiments, the amino acid residue at the position corresponding to position 50 in SEQ ID NO:4-7 (X_{13}) is an arginine or a glutamic acid.

[0028] In certain embodiments, the amino acid residue at the position corresponding to position 3 in SEQ ID NO:4-7 is an alanine and/or the amino acid residue at the position corresponding to position 6 in SEQ ID NO:4-7 is an aspartic acid. One of the amino acid residues at positions 3 and 6 may be an asparagine and in an alternative embodiment both amino acid residues at positions 3 and 6 may be asparagines.

[0029] In some embodiments the amino acid residue at the position corresponding to position 43 in SEQ ID NO:4-7 (X_{11}) is an alanine or a glutamic acid, such as an alanine. In specific embodiments, the amino acid residues at positions 9 and 11 in SEQ ID NO: 7 are alanine and lysine/glutamic acid respectively, while the amino acid residue at position 43 is alanine or glutamic acid.

[0030] In certain embodiments the amino acid residue residue at the position corresponding to position 28 in SEQ ID NO:4-7 (X_5) is an alanine or an asparagine, such as an alanine.

[0031] In some embodiments the amino acid residue at the position corresponding to position 40 in SEQ ID NO:4-7 (X_9) is selected from the group consisting of asparagine, alanine, glutamic acid and valine, or from the group consisting of glutamic acid and valine. In specific embodiments, the amino acid residues at positions 9 and 11 in SEQ ID NO: 7 are alanine and glutamic acid respectively, while the amino acid residue at position 40 is valine. Optionally, the amino acid residue at position 43 may then be alanine or glutamic acid.

[0032] In certain embodiments, the amino acid residue at the position corresponding to position 42 in SEQ ID NO:4-7 (X_{10}) is an alanine, lysine or arginine.

[0033] In some embodiments the amino acid residue at the position corresponding to position 18 in SEQ ID NO:4-7 (X_3) is a lysine or a histidine, such as a lysine.

[0034] In certain embodiments the amino acid residue at the position corresponding to position 33 in SEQ ID NO:4-7 (X_7) is a lysine or a serine, such as a lysine.

[0035] In some embodiments the amino acid residue at the position corresponding to position 37 in SEQ ID NO:4-7 (X_8) is a glutamic acid or an aspartic acid, such as a glutamic acid.

[0036] In certain embodiments the amino acid residue at the position corresponding to position 51 in SEQ ID NO:4-7 (X_{14}) is a tyrosine or a leucine, such as a tyrosine.

[0037] In some embodiments, the amino acid residue at the position corresponding to position 44 in SEQ ID NO:4-7 (X_{12}) is a leucine or an isoleucine. In specific embodiments, the amino acid residues at positions 9 and 11 in SEQ ID NO: 7 are alanine and lysine/glutamic acid respectively, while the amino acid residue at position 44 is isoleucine. Optionally, the amino acid residue at position 43 may then be alanine or glutamic acid.

[0038] In some embodiments, the amino acid residues at the positions corresponding to positions 1, 2, 3 and 4 or to positions 3, 4, 5 and 6 in SEQ ID NO: 4-7 have been deleted. In specific variants of these embodiments, the parental polypeptide is the C domain of Protein A (SEQ ID NO: 5). The effects of these deletions on the native C domain are described in US9018305 and US8329860, which are hereby incorporated by reference in their entireties.

[0039] In certain embodiments, the mutation in SEQ ID NO 4-7, such as in SEQ ID NO 7, is selected from the group consisting of:N1 1K; N11E; N11Y; N11T; N11F; N11L; N11W; N11I; N11M; N11V; N11A; N11H; N11R; N11E,Q32A; N11E,Q32E,Q40E; N11E,Q32E,K50R; Q9A,N11E,N43A; Q9A,N11E,N28A,N43A; Q9A,N11E,Q40V,A42K,N43E,L44I; Q9A,N11E,Q40V,A42K,N43A,L44I; N11K,H18K,S33K,D37E,A42R,N43A,L44I,K50R,L51Y; Q9A,N11E,N28A,Q40V,A42K,N43A,L44I; Q9A,N11K,H18K,S33K,D37E,A42R,N43A,L44I,K50R,L51Y; N11K, H18K, D37E, A42R, N43A, L44I; Q9A, N11K, H18K, D37E, A42R, N43A, L44I; Q9A, N11K, H18K, D37E, A42R, N43A, L44I, K50R; Q9A,N11K,H18K,D37E,A42R; Q9A,N11E,D37E,Q40V,A42K,N43A,L44I and Q9A,N11E,D37E,Q40V,A42R,N43A,L44I. These mutations provide particularly high alkaline stabilities. The mutation in SEQ ID NO 4-7, such as in SEQ ID NO 7, can also be selected from the group consisting of N11K; N11Y; N11F; N11L; N11W; N11I; N11M; N11V; N11A; N11H; N11R; Q9A,N11E,N43A; Q9A,N11E,N28A,N43A; Q9A,N11E,Q40V,A42K,N43E,L44I; Q9A,N11E,Q40V,A42K,N43A,L44I; Q9A,N11E,N28A,Q40V,A42K,N43A,L44I; N11K,H18K,S33K,D37E,A42R,N43A,L44I,K50R,L51Y; Q9A,N11K,H18K,S33K,D37E,A42R,N43A,L44I,K50R,L51Y; N11K, H18K, D37E, A42R, N43A, L44I; Q9A, N11K, H18K, D37E, A42R, N43A, L44I and Q9A, N11K, H18K, D37E, A42R, N43A, L44I, K50R.

[0040] In some embodiments, the polypeptide comprises or consists essentially of a sequence selected from the group consisting of: SEQ ID NO 8, SEQ ID NO 9, SEQ ID NO 10, SEQ ID NO 11, SEQ ID NO 12, SEQ ID NO 13, SEQ ID NO 14, SEQ ID NO 15, SEQ ID NO 16, SEQ ID NO 23, SEQ ID NO 24, SEQ ID NO 25, SEQ ID NO 26, SEQ ID NO 27, SEQ ID NO 28, SEQ ID NO 29, SEQ ID NO 36, SEQ ID NO 37, SEQ ID NO 38, SEQ ID NO 39, SEQ ID NO 40, SEQ ID NO 41, SEQ ID NO 42, SEQ ID NO 43, SEQ ID NO 44, SEQ ID NO 45, SEQ ID NO 46, SEQ ID NO 47, SEQ ID NO 48, SEQ ID NO 49 and SEQ ID NO 50. It may e.g. comprise or consist essentially of a sequence selected from the group consisting of: SEQ ID NO 8, SEQ ID NO 9, SEQ ID NO 10, SEQ ID NO 11, SEQ ID NO 16, SEQ ID NO 23, SEQ ID NO 24, SEQ ID NO 25, SEQ ID NO 26, SEQ ID NO 27, SEQ ID NO 28 and SEQ ID NO 29. It can also comprise or consist essentially of a sequence selected from the group consisting of: SEQ ID NO 8, SEQ ID NO 9, SEQ ID NO 10, SEQ ID

NO 11, SEQ ID NO 16, SEQ ID NO 23, SEQ ID NO 24, SEQ ID NO 25, SEQ ID NO 27, SEQ ID NO 28, SEQ ID NO 38, SEQ ID NO 40; SEQ ID NO 41; SEQ ID NO 42; SEQ NO 43, SEQ ID NO 44, SEQ ID NO 45, SEQ ID NO 46, SEQ ID NO 47 and SEQ ID NO 48. The polypeptide may e.g. be defined by a sequence selected from the groups above or from subsets of these groups, but it may also comprise additional amino acid residues at the N- and/or C-terminal end, e.g. a leader sequence at the N-terminal end and/or a tail sequence at the C-terminal end.

SEQ ID NO 8 Zvar(Q9A,N11E,N43A)

VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRNAF IQSLKDDPSQ SA**A**LLAEAKK LNDAQAPK

SEQ ID NO 9 Zvar(Q9A,N11E,N28A,N43A)
VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQR**A**AF IQSLKDDPSQ SA**A**LLAEAKK LNDAQAPK

SEQ ID NO 10 Zvar(Q9A,N11E,Q40V,A42K,N43E,L44I)
VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRNAF IQSLKDDPS**V** S**KEI**LAEAKK LNDAQAPK

SEQ ID NO 11 Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)
VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRNAF IQSLKDDPS**V** S**KAI**LAEAKK LNDAQAPK

SEQ ID NO 12 Zvar(N11E,Q32A)

VDAKFDKEQQ **E**AFYEILHLP NLTEEQRNAF I**A**SLKDDPSQ SANLLAEAKK LNDAQAPK

SEQ ID NO 13 Zvar(N11E)
VDAKFDKEQQ **E**AFYEILHLP NLTEEQRNAF IQSLKDDPSQ SANLLAEAKK LNDAQAPK

SEQ ID NO 14 Zvar(N11E,Q32E,Q40E)
VDAKFDKEQQ **E**AFYEILHLP NLTEEQRNAF I**E**SLKDDPS**E** SANLLAEAKK LNDAQAPK

SEQ ID NO 15 Zvar(N11E,Q32E,K50R)
VDAKFDKEQQ **E**AFYEILHLP NLTEEQRNAF I**E**SLKDDPSQ SANLLAEAK**R** LNDAQAPK

SEQ ID NO 16 Zvar(N11K)
VDAKFDKEQQ **K**AFYEILHLP NLTEEQRNAF IQSLKDDPSQ SANLLAEAKK LNDAQAPK

SEQ ID NO 23 Zvar(N11K,H18K,S33K,D37E,A42R,N43A,L44I,K50R,L51Y)
VDAKFDKEQQ **K**AFYEIL**K**LP NLTEEQRNAF IQ**K**LKD**E**PSQ S**RAI**LAEAK**R** **Y**NDAQAPK

SEQ ID NO 24 Zvar(Q9A,N11E,N28A,Q40V,A42K,N43A,L44I)
VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQR**A**AF IQSLKDDPS**V** S**KAI**LAEAKK LNDAQAPK

SEQ ID NO 25 Zvar(Q9A,N11K,H18K,S33K,D37E,A42R,N43A,L44I,K50R,L51Y)
VDAKFDKE**A**Q **K**AFYEIL**K**LP NLTEEQR**A**AF IQ**K**LKD**E**PSQ S**RAI**LAEAK**R** **Y**NDAQAPK

SEQ ID NO 26 Zvar(N11K, H18K, D37E, A42R, N43A, L44I)
VDAKFDKEQQ **K**AFYEIL**K**LP NLTEEQRNAF IQSLKD**E**PSQ S**RAI**LAEAKK LNDAQAPK

SEQ ID NO 27 Zvar(Q9A, N11K, H18K, D37E, A42R, N43A, L44I)
VDAKFDKE**A**Q **K**AFYEIL**K**LP NLTEEQRNAF IQSLKD**E**PSQ S**RAI**LAEAKK LNDAQAPK

SEQ ID NO 28 Zvar(Q9A, N11K, H18K, D37E, A42R, N43A, L44I, K50R)
VDAKFDKE**A**Q **K**AFYEIL**K**LP NLTEEQRNAF IQSLKD**E**PSQ S**RAI**LAEAK**R** LNDAQAPK

SEQ ID NO 29 Zvar(Q9A,N11K,H18K,D37E,A42R)
VDAKFDKE**A**Q **K**AFYEIL**K**LP NLTEEQRNAF IQSLKD**E**PSQ S**R**NLLAEAKK LNDAQAPK

SEQ ID NO 36 B(Q9A,N11E,Q40V,A42K,N43A,L44I)
ADNKFNKE**A**Q **E**AFYEILHLP NLNEEQRNGF IQSLKDDPS**V** S**KAI**LAEAKK LNDAQAPK

SEQ ID NO 37 C(Q9A,N11E,E43A)
ADNKFNKE**A**Q **E**AFYEILHLP NLTEEQRNGF IQSLKDDPSV SK**A**ILAEAKK LNDAQAPK

SEQ ID NO 38 Zvar(N11Y)
VDAKFDKEQQ **Y**AFYEILHLP NLTEEQRNAF IQSLKDDPSQ SANLLAEAKK LNDAQAPK

SEQ ID NO 39 Zvar(N11T)
VDAKFDKEQQ **T**AFYEILHLP NLTEEQRNAF IQSLKDDPSQ SANLLAEAKK LNDAQAPK

SEQ ID NO 40 Zvar(N11F)
VDAKFDKEQQ **F**AFYEILHLP NLTEEQRNAF IQSLKDDPSQ SANLLAEAKK LNDAQAPK

SEQ ID NO 41 Zvar(N11L)
VDAKFDKEQQ **L**AFYEILHLP NLTEEQRNAF IQSLKDDPSQ SANLLAEAKK LNDAQAPK

SEQ ID NO 42 Zvar(N11W)
VDAKFDKEQQ **W**AFYEILHLP NLTEEQRNAF IQSLKDDPSQ SANLLAEAKK LNDAQAPK

SEQ ID NO 43 Zvar(N11I)
VDAKFDKEQQ **I**AFYEILHLP NLTEEQRNAF IQSLKDDPSQ SANLLAEAKK LNDAQAPK

SEQ ID NO 44 Zvar(N11M)
VDAKFDKEQQ **M**AFYEILHLP NLTEEQRNAF IQSLKDDPSQ SANLLAEAKK LNDAQAPK

SEQ ID NO 45 Zvar(N11V)
VDAKFDKEQQ **V**AFYEILHLP NLTEEQRNAF IQSLKDDPSQ SANLLAEAKK LNDAQAPK

SEQ ID NO 46 Zvar(N11A)
VDAKFDKEQQ **A**AFYEILHLP NLTEEQRNAF IQSLKDDPSQ SANLLAEAKK LNDAQAPK

SEQ ID NO 47 Zvar(N11H)
VDAKFDKEQQ **H**AFYEILHLP NLTEEQRNAF IQSLKDDPSQ SANLLAEAKK LNDAQAPK

SEQ ID NO 48 Zvar(N11R)
VDAKFDKEQQ **R**AFYEILHLP NLTEEQRNAF IQSLKDDPSQ SANLLAEAKK LNDAQAPK

SEQ ID NO 49 Zvar(Q9A,N11E,D37E,Q40V,A42K,N43A,L44I)
VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRNAF IQSLKD**E**PS**V** S**KAI**LAEAKK LNDAQAPK

SEQ ID NO 50 Zvar(Q9A,N11E,D37E,Q40V,A42R,N43A,L44I)
VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRNAF IQSLKD**E**PS**V** S**RAI**LAEAKK LNDAQAPK

[0041] In a second aspect the present invention discloses a multimer comprising, or consisting essentially of, a plurality of polypeptide units as defined by any embodiment disclosed above. The multimer can e.g. be a dimer, a trimer, a tetramer, a pentamer, a hexamer, a heptamer, an octamer or a nonamer. It can be a homomultimer, where all the units in the multimer are identical or it can be a heteromultimer, where at least one unit differs from the others. Advantageously, all the units in the multimer are alkali stable, such as by comprising the mutations disclosed above. The polypeptides can be linked to each other directly by peptide bonds between the C-terminal and N-terminal ends of the polypeptides. Alternatively, two or more units in the multimer can be linked by linkers comprising oligomeric or polymeric species, such as elements comprising up to 15 or 30 amino acids, such as 1-5, 1-10 or 5-10 amino acids. This is the case in particular for mutations of SEQ ID NO 51 and 52 and for the SEQ ID NO 53 polypeptide, where specific examples of linkers can e.g. be VDAKFD or ADNKFN, such as VDAKFD. The nature of such a linker should preferably not destabilize the spatial conformation of the protein units. This can e.g. be achieved by avoiding the presence of proline in the linkers. Furthermore, said linker should preferably also be sufficiently stable in alkaline environments not to impair the properties of the mutated protein units. For this purpose, it is advantageous if the linkers do not contain asparagine. It can additionally be advantageous if the linkers do not contain glutamine. The multimer may further at the N-terminal end comprise a plurality of amino acid residues e.g. originating from the cloning process or constituting a residue from a cleaved off signaling sequence. The number of additional amino acid residues may e.g. be 15 or less, such as 10 or less or 5 or less. As a specific example, the multimer may comprise an AQ sequence at the N-terminal end.

[0042] In certain embodiments, the multimer may comprise, or consist essentially, of a sequence selected from the group consisting of: SEQ ID NO 17, SEQ ID NO 18, SEQ ID NO 19, SEQ ID NO 20, SEQ ID NO 30, SEQ ID NO 31, SEQ ID NO 32, SEQ ID NO 33, SEQ ID NO 34 and SEQ ID NO 35. These sequences are listed below and named as Parent(Mutations)n, where n is the number of monomer units in a multimer.

SEQ ID NO 17 Zvar(Q9A,N11E,N43A)4
AQGT VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSQ SAALLAEAKK LNDAQAPK VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSQ SAALLAEAKK LNDAQAPK VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSQ SAALLAEAKK LNDAQAPK VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSQ SAALLAEAKK LNDAQAPKC

SEQ ID NO 18 Zvar(Q9A,N11E,N28A,N43A)4
AQGT VDAKFDKEAQ EAFYEILHLP NLTEEQRAAF IQSLKDDPSQ SAALLAEAKK LNDAQAPK VDAKFDKEAQ EAFYEILHLP NLTEEQRAAF IQSLKDDPSQ SAALLAEAKK LNDAQAPK VDAKFDKEAQ EAFYEILHLP NLTEEQRAAF IQSLKDDPSQ SAALLAEAKK LNDAQAPK VDAKFDKEAQ EAFYEILHLP NLTEEQRAAF IQSLKDDPSQ SAALLAEAKK LNDAQAPKC

SEQ ID NO 19 Zvar(Q9A,N11E,Q40V,A42K,N43E,L44I)4
AQGT VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKEILAEAKK LNDAQAPK VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKEILAEAKK LNDAQAPK VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKEILAEAKK LNDAQAPK VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKEILAEAKK LNDAQAPKC

SEQ ID NO 20 Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)4
AQGT VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LNDAQAPK VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LNDAQAPK VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LNDAQAPK VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LNDAQAPKC

SEQ ID NO 30 Zvar(N11K,H18K,S33K,D37E,A42R,N43A,L44I,K50R,L51Y)4
AQGT VDAKFDKEQQ KAFYEILKLP NLTEEQRNAF IQKLKDEPSQ SRAILAEAKR YNDAQAPK VDAKFDKEQQ KAFYEILKLP NLTEEQRNAF IQKLKDEPSQ SRAILAEAKR YNDAQAPK VDAKFDKEQQ KAFYEILKLP NLTEEQRNAF IQKLKDEPSQ SRAILAEAKR YNDAQAPK VDAKFDKEQQ KAFYEILKLP NLTEEQRNAF IQKLKDEPSQ SRAILAEAKR YNDAQAPKC

SEQ ID NO 31 Zvar(Q9A,N11K,H18K,D37E,A42R)4
AQGT VDAKFDKEAQ KAFYEILKLP NLTEEQRNAF IQSLKDEPSQ SRNLLAEAKK LNDAQAPK VDAKFDKEAQ KAFYEILKLP NLTEEQRNAF IQSLKDEPSQ SRNLLAEAKK LNDAQAPK VDAKFDKEAQ KAFYEILKLP NLTEEQRNAF

IQSLKDEPSQ SRNLLAEAKK LNDAQAPK VDAKFDKEAQ KAFYEILKLP NLTEEQRNAF IQSLKDEPSQ SRNLLAEAKK LNDAQAPKC

SEQ ID NO 32 Zvar(Q9A,N11E,N28A,Q40V,A42K,N43A,L44I)4
AQGT VDAKFDKEAQ EAFYEILHLP NLTEEQRAAF IQSLKDDPSV SKAILAEAKK LNDAQAPK VDAKFDKEAQ EAFYEILHLP NLTEEQRAAF IQSLKDDPSV SKAILAEAKK LNDAQAPK VDAKFDKEAQ EAFYEILHLP NLTEEQRAAF IQSLKDDPSV SKAILAEAKK LNDAQAPK VDAKFDKEAQ EAFYEILHLP NLTEEQRAAF IQSLKDDPSV SKAILAEAKK LNDAQAPKC

SEQ ID NO 33 Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)6
AQGT VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LNDAQAPK VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LNDAQAPK VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LNDAQAPK VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LNDAQAPK VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LNDAQAPK VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LNDAQAPKC

SEQ ID NO 34 Zvar(Q9A,N11E,D37E,Q40V,A42K,N43A,L44I)4
AQGT VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRNAF IQSLKD**E**PS**V** S**KAI**LAEAKK LNDAQAPK VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRNAF IQSLKD**E**PS**V** S**KAI**LAEAKK LNDAQAPK VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRNAF IQSLKD**E**PS**V** S**KAI**LAEAKK LNDAQAPK VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRNAF IQSLKD**E**PS**V** S**KAI**LAEAKK LNDAQAPKC

SEQ ID NO 35 Zvar(Q9A,N11E,D37E,Q40V,A42R,N43A,L44I)4
AQGT VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRNAF IQSLKD**E**PS**V** S**RAI**LAEAKK LNDAQAPK VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRNAF IQSLKD**E**PS**V** S**RAI**LAEAKK LNDAQAPK VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRNAF IQSLKD**E**PS**V** S**RAI**LAEAKK LNDAQAPK VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRNAF IQSLKD**E**PS**V** S**RAI**LAEAKK LNDAQAPKC

[0043] In some embodiments, the polypeptide and/or multimer, as disclosed above, further comprises at the C-terminal or N-terminal end one or more coupling elements, selected from the group consisting of one or more cysteine residues, a plurality of lysine residues and a plurality of histidine residues. The coupling element(s) may also be located within 1-5 amino acid residues, such as within 1-3 or 1-2 amino acid residues from the C-terminal or N-terminal end. The coupling element may e.g. be a single cysteine at the C-terminal end. The coupling element(s) may be directly linked to the C- or N-terminal end, or it/they may be linked via a stretch comprising up to 15 amino acids, such as 1-5, 1-10 or 5-10 amino acids. This stretch should preferably also be sufficiently stable in alkaline environments not to impair the properties of the mutated protein. For this purpose, it is advantageous if the stretch does not contain asparagine. It can additionally be advantageous if the stretch does not contain glutamine. An advantage of having a C-terminal cysteine is that endpoint coupling of the protein can be achieved through reaction of the cysteine thiol with an electrophilic group on a support. This provides excellent mobility of the coupled protein which is important for the binding capacity.

[0044] The alkali stability of the polypeptide or multimer can be assessed by coupling it to an SPR chip, e.g. to Biacore CM5 sensor chips as described in the examples, using e.g. NHS- or maleimide coupling chemistries, and measuring the immunoglobulin-binding capacity of the chip, typically using polyclonal human IgG, before and after incubation in alkaline solutions at a specified temperature, e.g. 22 +/- 2 °C. The incubation can e.g. be performed in 0.5 M NaOH for a number of 10 min cycles, such as 100, 200 or 300 cycles. The IgG capacity of the matrix after 100 10 min incubation cycles in 0.5 M NaOH at 22 +/- 2 °C can be at least 55, such as at least 60, at least 80 or at least 90% of the IgG capacity before the incubation. Alternatively, the remaining IgG capacity after 100 cycles for a particular mutant measured as above can be compared with the remaining IgG capacity for the parental polypeptide/multimer. In this case, the remaining IgG capacity for the mutant may be at least 105%, such as at least 110%, at least 125%, at least 150% or at least 200% of the parental polypeptide/multimer.

[0045] In a third aspect the present invention discloses a nucleic acid encoding a polypeptide or multimer according to any embodiment disclosed above. Thus, the invention encompasses all forms of the present nucleic acid sequence such as the RNA and the DNA encoding the polypeptide or multimer. The invention embraces a vector, such as a plasmid, which in addition to the coding sequence comprises the required signal sequences for expression of the polypeptide or multimer according the invention. In one embodiment, the vector comprises nucleic acid encoding a multimer according to the invention, wherein the separate nucleic acids encoding each unit may have homologous or heterologous DNA sequences.

[0046] In a fourth aspect the present invention discloses an expression system, which comprises, a nucleic acid or a vector as disclosed above. The expression system may e.g. be a gram-positive or gram-negative prokaryotic host cell system, e.g. *E.coli* or *Bacillus sp.* which has been modified to express the present polypeptide or multimer. In an alternative embodiment, the expression system is a eukaryotic host cell system, such as a yeast, e.g. *Pichia pastoris* or *Saccharomyces cerevisiae,* or mammalian cells, e.g. CHO cells.

[0047] In a fifth aspect, the present invention discloses a separation matrix, wherein a plurality of polypeptides or multimers according to any embodiment disclosed above have been coupled to a solid support. Such a matrix is useful for separation of immunoglobulins or other Fc-containing proteins and, due to the improved alkali stability of the polypeptides/multimers, the matrix will withstand highly alkaline conditions during cleaning, which is essential for long-term repeated use in a bioprocess separation setting. The alkali stability of the matrix can be assessed by measuring the immunoglobulin-binding capacity, typically using polyclonal human IgG, before and after incubation in alkaline solutions at a specified temperature, e.g. 22 +/- 2 °C. The incubation can e.g. be performed in 0.5 M or 1.0 M NaOH for a number of 15 min cycles, such as 100, 200 or 300 cycles, corresponding to a total incubation time of 25, 50 or 75 h. The IgG capacity of the matrix after 96-100 15 min incubation cycles or a total incubation time of 24 or 25 h in 0.5 M NaOH at 22 +/- 2 °C can be at least 80, such as at least 85, at least 90 or at least 95% of the IgG capacity before the incubation. The capacity of the matrix after a total incubation time of 24 h in 1.0 M NaOH at 22 +/- 2 °C can be at least 70, such as at least 80 or at least 90% of the IgG capacity before the incubation.

[0048] As the skilled person will understand, the expressed polypeptide or multimer should be purified to an appropriate extent before being immobilized to a support. Such purification methods are well known in the field, and the immobilization of protein-based ligands to supports is easily carried out using standard methods. Suitable methods and supports will be discussed below in more detail.

[0049] The solid support of the matrix according to the invention can be of any suitable well-known kind. A conventional affinity separation matrix is often of organic nature and based on polymers that expose a hydrophilic surface to the aqueous media used, i.e. expose hydroxy (-OH), carboxy (-COOH), carboxamido (-$CONH_2$, possibly in N- substituted forms), amino (-NH_2, possibly in substituted form), oligo- or polyethylenoxy groups on their external and, if present, also on internal surfaces. The solid support can suitably be porous. The porosity can be expressed as a Kav or Kd value (the fraction of the pore volume available to a probe molecule of a particular size) measured by inverse size exclusion chromatography, e.g. according to the methods described in Gel Filtration Principles and Methods, Pharmacia LKB Biotechnology 1991, pp 6-13. By definition, both Kd and Kav values always lie within the range 0 - 1. The Kav value can advantageously be 0.6 - 0.95, e.g. 0.7 - 0.90 or 0.6 - 0.8, as measured with dextran of Mw 110 kDa as a probe molecule.

An advantage of this is that the support has a large fraction of pores able to accommodate both the polypeptides/multimers of the invention and immunoglobulins binding to the polypeptides/multimers and to provide mass transport of the immunoglobulins to and from the binding sites.

[0050] The polypeptides or multimers may be attached to the support via conventional coupling techniques utilising e.g. thiol, amino and/or carboxy groups present in the ligand. Bisepoxides, epichlorohydrin, CNBr, N-hydroxysuccinimide (NHS) etc are well-known coupling reagents. Between the support and the polypeptide/multimer, a molecule known as a spacer can be introduced, which improves the availability of the polypeptide/multimer and facilitates the chemical coupling of the polypeptide/multimer to the support. Depending on the nature of the polypeptide/multimer and the coupling conditions, the coupling may be a multipoint coupling (e.g. via a plurality of lysines) or a single point coupling (e.g. via a single cysteine). Alternatively, the polypeptide/multimer may be attached to the support by non-covalent bonding, such as physical adsorption or biospecific adsorption.

[0051] In some embodiments the matrix comprises 5 - 25, such as 5-20 mg/ml, 5 - 15 mg/ml, 5 - 11 mg/ml or 6 - 11 mg/ml of the polypeptide or multimer coupled to the support. The amount of coupled polypeptide/multimer can be controlled by the concentration of polypeptide/multimer used in the coupling process, by the activation and coupling conditions used and/or by the pore structure of the support used. As a general rule the absolute binding capacity of the matrix increases with the amount of coupled polypeptide/multimer, at least up to a point where the pores become significantly constricted by the coupled polypeptide/multimer. The relative binding capacity per mg coupled polypeptide/multimer will decrease at high coupling levels, resulting in a cost-benefit optimum within the ranges specified above.

[0052] In certain embodiments the polypeptides or multimers are coupled to the support via thioether bonds. Methods for performing such coupling are well-known in this field and easily performed by the skilled person in this field using standard techniques and equipment. Thioether bonds are flexible and stable and generally suited for use in affinity chromatography. In particular when the thioether bond is via a terminal or near-terminal cysteine residue on the polypeptide or multimer, the mobility of the coupled polypeptide/multimer is enhanced which provides improved binding capacity and binding kinetics. In some embodiments the polypeptide/multimer is coupled via a C-terminal cysteine provided on the protein as described above. This allows for efficient coupling of the cysteine thiol to electrophilic groups, e.g. epoxide groups, halohydrin groups etc. on a support, resulting in a thioether bridge coupling.

[0053] In certain embodiments the support comprises a polyhydroxy polymer, such as a polysaccharide. Examples of polysaccharides include e.g. dextran, starch, cellulose, pullulan, agar, agarose etc. Polysaccharides are inherently hydrophilic with low degrees of nonspecific interactions, they provide a high content of reactive (activatable) hydroxyl groups and they are generally stable towards alkaline cleaning solutions used in bioprocessing.

[0054] In some embodiments the support comprises agar or agarose. The supports used in the present invention can easily be prepared according to standard methods, such as inverse suspension gelation (S Hjertén: Biochim Biophys Acta 79(2), 393-398 (1964). Alternatively, the base matrices are commercially available products, such as crosslinked agarose beads sold under the name of SEPHAROSE™ FF (GE Healthcare). In an embodiment, which is especially advantageous for large-scale separations, the support has been adapted to increase its rigidity using the methods described in US6602990 or US7396467, which are hereby incorporated by reference in their entirety, and hence renders the matrix more suitable for high flow rates.

[0055] In certain embodiments the support, such as a polysaccharide or agarose support, is crosslinked, such as with hydroxyalkyl ether crosslinks. Crosslinker reagents producing such crosslinks can be e.g. epihalohydrins like epichlorohydrin, diepoxides like butanediol diglycidyl ether, allylating reagents like allyl halides or allyl glycidyl ether. Crosslinking is beneficial for the rigidity of the support and improves the chemical stability. Hydroxyalkyl ether crosslinks are alkali stable and do not cause significant nonspecific adsorption.

[0056] Alternatively, the solid support is based on synthetic polymers, such as polyvinyl alcohol, polyhydroxyalkyl acrylates, polyhydroxyalkyl methacrylates, polyacrylamides, polymethacrylamides etc. In case of hydrophobic polymers, such as matrices based on divinyl and monovinyl-substituted benzenes, the surface of the matrix is often hydrophilised to expose hydrophilic groups as defined above to a surrounding aqueous liquid. Such polymers are easily produced according to standard methods, see e.g. "Styrene based polymer supports developed by suspension polymerization" (R Arshady: Chimica e L'Industria 70(9), 70-75 (1988)). Alternatively, a commercially available product, such as SOURCE™ (GE Healthcare) is used. In another alternative, the solid support according to the invention comprises a support of inorganic nature, e.g. silica, zirconium oxide etc.

[0057] In yet another embodiment, the solid support is in another form such as a surface, a chip, capillaries, or a filter (e.g. a membrane or a depth filter matrix).

[0058] As regards the shape of the matrix according to the invention, in one embodiment the matrix is in the form of a porous monolith. In an alternative embodiment, the matrix is in beaded or particle form that can be porous or non-porous. Matrices in beaded or particle form can be used as a packed bed or in a suspended form. Suspended forms include those known as expanded beds and pure suspensions, in which the particles or beads are free to move. In case of monoliths, packed bed and expanded beds, the separation procedure commonly follows conventional chromatography with a concentration gradient. In case of pure suspension, batchwise mode will be used.

[0059] In a sixth aspect, the present invention discloses a method of isolating an immunoglobulin, wherein a separation matrix as disclosed above is used.

[0060] In certain embodiments, the method comprises the steps of:

a) contacting a liquid sample comprising an immunoglobulin with a separation matrix as disclosed above,
b) washing said separation matrix with a washing liquid,
c) eluting the immunoglobulin from the separation matrix with an elution liquid, and
d) cleaning the separation matrix with a cleaning liquid, which can alternatively be called a cleaning-in-place (CIP) liquid, e.g. with a contact (incubation) time of at least 10 min.

The method may also comprise steps of, before step a), providing an affinity separation matrix according to any of the embodiments described above and providing a solution comprising an immunoglobulin and at least one other substance as a liquid sample and of, after step c), recovering the eluate and optionally subjecting the eluate to further separation steps, e.g. by anion or cation exchange chromatography, multimodal chromatography and/or hydrophobic interaction chromatography. Suitable compositions of the liquid sample, the washing liquid and the elution liquid, as well as the general conditions for performing the separation are well known in the art of affinity chromatography and in particular in the art of Protein A chromatography. The liquid sample comprising an Fc-containing protein and at least one other substance may comprise host cell proteins (HCP), such as CHO cell, E Coli or yeast proteins. Contents of CHO cell and E Coli proteins can conveniently be determined by immunoassays directed towards these proteins, e.g. the CHO HCP or E Coli HCP ELISA kits from Cygnus Technologies. The host cell proteins or CHO cell/E Coli proteins may be desorbed during step b).

[0061] The elution may be performed by using any suitable solution used for elution from Protein A media. This can e.g. be a solution or buffer with pH 5 or lower, such as pH 2.5 - 5 or 3 - 5. It can also in some cases be a solution or buffer with pH 11 or higher, such as pH 11 - 14 or pH 11 - 13. In some embodiments the elution buffer or the elution buffer gradient comprises at least one mono- di- or trifunctional carboxylic acid or salt of such a carboxylic acid. In certain embodiments the elution buffer or the elution buffer gradient comprises at least one anion species selected from the group consisting of acetate, citrate, glycine, succinate, phosphate, and formiate.

[0062] In some embodiments, the cleaning liquid is alkaline, such as with a pH of 13 - 14. Such solutions provide efficient cleaning of the matrix, in particular at the upper end of the interval

[0063] In certain embodiments, the cleaning liquid comprises 0.1 - 2.0 M NaOH or KOH, such as 0.5 - 2.0 or 0.5 - 1.0 M NaOH or KOH. These are efficient cleaning solutions, and in particular so when the NaOH or KOH concentration is above 0.1 M or at least 0.5 M. The high stability of the polypeptides of the invention enables the use of such strongly alkaline solutions.

[0064] The method may also include a step of sanitizing the matrix with a sanitization liquid, which may e.g. comprise a peroxide, such as hydrogen peroxide and/or a peracid, such as peracetic acid or performic acid.

[0065] In some embodiments, steps a) - d) are repeated at least 10 times, such as at least 50 times, 50 - 200, 50-300 or 50-500 times. This is important for the process economy in that the matrix can be re-used many times.

[0066] Steps a) - c) can also be repeated at least 10 times, such as at least 50 times, 50 - 200, 50-300 or 50-500 times, with step d) being performed after a plurality of instances of step c), such that step d) is performed at least 10 times, such as at least 50 times. Step d) can e.g. be performed every second to twentieth instance of step c).

Examples

Mutagenesis of protein

[0067] Site-directed mutagenesis was performed by a two-step PCR using oligonucleotides coding for the mutations. As template a plasmid containing a single domain of either Z, B or C was used. The PCR fragments were ligated into an E. coli expression vector. DNA sequencing was used to verify the correct sequence of inserted fragments.

[0068] To form multimers of mutants an Acc I site located in the starting codons (GTA GAC) of the B, C or Z domain was used, corresponding to amino acids VD. The vector for the monomeric domain was digested with Acc I and phosphatase treated. Acc I sticky-ends primers were designed, specific for each variant, and two overlapping PCR products were generated from each template. The PCR products were purified and the concentration was estimated by comparing the PCR products on a 2% agarose gel. Equal amounts of the pair wise PCR products were hybridized (90°C -> 25°C in 45min) in ligation buffer. The resulting product consists approximately to ¼ of fragments likely to be ligated into an Acc I site (correct PCR fragments and/or the digested vector). After ligation and transformation colonies were PCR screened to identify constructs containing the desired mutant. Positive clones were verified by DNA sequencing.

Construct expression and purification

[0069] The constructs were expressed in the bacterial periplasm by fermentation of *E. coli* K12 in standard media. After fermentation the cells were heat-treated to release the periplasm content into the media. The constructs released into the medium were recovered by microfiltration with a membrane having a 0.2 μm pore size.

[0070] Each construct, now in the permeate from the filtration step, was purified by affinity. The permeate was loaded onto a chromatography medium containing immobilized IgG (IgG Sepharose 6FF, GE Healthcare). The loaded product was washed with phosphate buffered saline and eluted by lowering the pH.

The elution pool was adjusted to a neutral pH (pH 8) and reduced by addition of dithiothreitol. The sample was then loaded onto an anion exchanger. After a wash step the construct was eluted in a NaCl gradient to separate it from any contaminants. The elution pool was concentrated by ultrafiltration to 40-50 mg/ml. It should be noted that the successful affinity purification of a construct on an immobilized IgG medium indicates that the construct in question has a high affinity to IgG.

[0071] The purified ligands were analyzed with RPC LC-MS to determine the purity and to ascertain that the molecular weight corresponded to the expected (based on the amino acid sequence).

Example 1

[0072] The purified monomeric ligands listed in Table 1, further comprising an AQGT leader sequence at the N-terminus and a cysteine at the C terminus, were immobilized on Biacore CM5 sensor chips (GE Healthcare, Sweden), using the amine coupling kit of GE Healthcare (for carbodiimide coupling of amines on the carboxymethyl groups on the chip) in an amount sufficient to give a signal strength of about 200-1500 RU in a Biacore surface plasmon resonance (SPR) instrument (GE Healthcare, Sweden) . To follow the IgG binding capacity of the immobilized surface 1mg/ml human polyclonal IgG (Gammanorm) was flowed over the chip and the signal strength (proportional to the amount of binding) was noted. The surface was then cleaned-in-place (CIP), i.e. flushed with 500mM NaOH for 10 minutes at room temperature (22 +/- 2°C). This was repeated for 96-100 cycles and the immobilized ligand alkaline stability was followed as the remaining IgG binding capacity (signal strength) after each cycle. The results are shown in Table 1 and indicate that at least the ligands Zvar(N11K)1, Zvar(N11E)1, Zvar(N11Y)1, Zvar(N11T)1, Zvar(N11F)1, Zvar(N11L)1, Zvar(N11W)1, ZN11I)1, Zvar(N11M)1, Zvar(N11V)1, Zvar(N11A)1, Zvar(N11H1), Zvar(N11R)1, Zvar(N11E,Q32A)1, Zvar(N11E,Q32E,Q40E)1 and Zvar(N11E,Q32E,K50R)1, Zvar(Q9A,N1 1E,N43A)1, Zvar(Q9A,N11E,N28A,N43A)1, Zvar(Q9A,N1 1E,Q40V,A42K,N43E,L44I)1, Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)1, Zvar(Q9A,N11E,N28A,Q40V,A42K,N43A,L44I)1, Zvar(N11K,H18K,S33K,D37E,A42R,N43A,L44I,K50R,L51Y)1, Zvar(Q9A,N11K,H18K,S33K,D37E,A42R,N43A,L44I,K50R,L51Y)1, Zvar(N11K, H18K, D37E, A42R, N43A, L44I)1, Zvar(Q9A, N11K, H18K, D37E, A42R, N43A, L44I)1 and Zvar(Q9A, N11K, H18K, D37E, A42R, N43A, L44I, K50R)1 have an improved alkali stability compared to the parental structure Zvar1, used as the reference. Further, the ligands B(Q9A,N11E,Q40V,A42K,N43A,L44I)1 and C(Q9A,N11E,E43A)1 have an improved stability compared to the parental B and C domains, used as references.

Table 1. Monomeric ligands, evaluated by Biacore (0.5 M NaOH).

Ligand	**Sequence**	**Capacity after 96-100 cycles**	**Reference capacity after 96-100 cycles**	**Capacity relative to reference**
Zvar(N11E,Q32A)1	SEQ ID NO 12	57%	55%	1.036
Zvar(N11E)1	SEQ ID NO 13	59%	55%	1.073
Zvar(N1 1E,Q32E,Q40E)1	SEQ ID NO 14	52%	51%	1.020
Zvar(N1 1E,Q32E,K50R)1	SEQ ID NO 15	53%	51%	1.039
Zvar(N11K) 1	SEQ ID NO 16	62%	49%	1.270
Zvar(N11Y)1	SEQ ID NO 38	55%	46%	1.20

(continued)

Ligand	Sequence	Capacity after 96-100 cycles	Reference capacity after 96-100 cycles	Capacity relative to reference
Zvar(N11T)1	SEQ ID NO 39	50%	46%	1.09
Zvar(N11F)1	SEQ ID NO 40	55%	46%	1.20
Zvar(N11L)1	SEQ ID NO 41	57%	47%	1.21
Zvar(N11W)1	SEQ ID NO 42	57%	47%	1.21
Zvar(N11I)1	SEQ ID NO 43	57%	47%	1.21
Zvar(N11M)1	SEQ ID NO 44	58%	46%	1.26
Zvar(N11V)1	SEQ ID NO 45	56%	46%	1.22
Zvar(N11A) 1	SEQ ID NO 46	58%	46%	1.26
Zvar(N11H) 1	SEQ ID NO 47	57%	46%	1.24
Zvar(N11R)1	SEQ ID NO 48	59%	46%	1.28
Zvar(Q9A,N11E,N43A)1	SEQ ID NO 8	70%	47%	1.49
Zvar(Q9A,N1 1E,N28A,N43A) 1	SEQ ID NO 9	68%	47%	1.45
Zvar(Q9A,N11E,Q40V,A42K, N43E,L44I)1	SEQ ID NO 10	67%	47%	1.43
Zvar(Q9A,N11E,Q40V,A42K, N43A,L44I) 1	SEQ ID NO 11	66%	47%	1.40
Zvar(Q9A,N11E,N28A,Q40V, A42K,N43A,L44I)1	SEQ ID NO 24	65%	48%	1.35
Zvar(N11K,H18K,S33K,D37E, A42R,N43A,L44I,K50R,L51Y) 1	SEQ ID NO 23	67%	46%	1.46
Zvar(Q9A,N 1 1K,H18K,S33K, D37E,A42R,N43A,L44I,K50R, L5 1Y)1	SEQ ID NO 25	59%	46%	1.28
Zvar(N11K, H18K, D37E, A42R, N43A, L44I)1	SEQ ID NO 26	59%	45%	1.31
Zvar(Q9A, N11K, H18K, D37E, A42R, N43A, L44I)1	SEQ ID NO 27	63%	45%	1.40
Zvar(Q9A, N11K, H18K, D37E, A42R, N43A, L44I, K50R)1	SEQ ID NO 28	67%	45%	1.49
B(Q9A,N11E,Q40V,A42K, N43A,L44I)1	SEQ ID NO 36	39%	35%	1.11

(continued)

Ligand	Sequence	Capacity after 96-100 cycles	Reference capacity after 96-100 cycles	Capacity relative to reference
C(Q9A,N11E,E43A)1	SEQ ID NO 37	60%	49%	1.22

Example 2

[0073] The purified tetrameric and hexameric ligands listed in Table 2 were immobilized on Biacore CM5 sensor chips (GE Healthcare, Sweden), using the amine coupling kit of GE Healthcare (for carbodiimide coupling of amines on the carboxymethyl groups on the chip) in an amount sufficient to give a signal strength of about 200-1500 RU in a Biacore instrument (GE Healthcare, Sweden). To follow the IgG binding capacity of the immobilized surface 1mg/ml human polyclonal IgG (Gammanorm) was flowed over the chip and the signal strength (proportional to the amount of binding) was noted. The surface was then cleaned-in-place (CIP), i.e. flushed with 500mM NaOH for 10 minutes at room temperature (22 +/- 2°C). This was repeated for 300 cycles and the immobilized ligand alkaline stability was followed as the remaining IgG binding capacity (signal strength) after each cycle. The results are shown in Table 2 and in Fig. 2 and indicate that at least the ligands Zvar(Q9A,N1 1E,N43A)4, Zvar(Q9A,N11E,N28A,N43A)4, Zvar(Q9A,N1 1E,Q40V,A42K,N43E,L44I)4 and Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)4, Zvar(Q9A,N11E,D37E,Q40V,A42K,N43A,L44I)4 and Zvar(Q9A,N11E,D37E,Q40V,A42R,N43A,L44I)4 have an improved alkali stability compared to the parental structure Zvar4, which was used as a reference. The hexameric ligand Zvar(Q9A,N1 1E,Q40V,A42K,N43A,L44I)6 also has improved alkali stability compared to the parental structure Zvar6, used as a reference.

Table 2. Tetrameric and hexameric ligands, evaluated by Biacore (0.5M NaOH).

Ligand	SEQ ID NO:	Remaining capacity 100 cycles (%)	Capacity relative to ref. 100 cycles	Remaining capacity 200 cycles (%)	Capacity relative to ref. 200 cycles	Remaining capacity 300 cycles (%)	Capacity relative to ref. 300 cycles
Zvar4	21	67	1	36	1	16	1
Zvar(Q9A, N11E,N43A)4	17	81	1.21	62	1.72	41	2.56
Zvar(Q9A, N11E,N28A,N 43A)4	18	80	1.19	62	1.72	42	2.62
Zvar(Q9A, N11E,Q40V,A 42K,N43E, L44I)4	19	84	1.25	65	1.81	48	3.00
Zvar(Q9A, N11E,Q40V,A 42K,N43A, L44I)4	20	90	1.34	74	2.06	57	3.56
Zvar(Q9A,N1 1E,N28A,Q 40V,A42K, N43A,L44I)4	32	84	1.24	Not tested	Not tested	Not tested	Not tested
Zvar(Q9A, N11E,Q40V,A 42K,N43A, L44I)6	33	87	1.30	Not tested	Not tested	Not tested	Not tested

(continued)

Ligand	SEQ ID NO:	Remaining capacity 100 cycles (%)	Capacity relative to ref. 100 cycles	Remaining capacity 200 cycles (%)	Capacity relative to ref. 200 cycles	Remaining capacity 300 cycles (%)	Capacity relative to ref. 300 cycles
Zvar(Q9A, N11E,D37E,Q 40V,A42K, N43A,L44I)4	34	81	1.13	Not tested	Not tested	Not tested	Not tested
Zvar(Q9A, N11E,D37E,Q 40V,A42R, N43A,L44I)4	35	84	1.17	Not tested	Not tested	Not tested	Not tested

Example 3

[0074] Example 2 was repeated with 100 CIP cycles of three ligands using 1 M NaOH instead of 500 mM as in Example 2. The results are shown in Table 3 and show that all three ligands have an improved alkali stability also in 1M NaOH, compared to the parental structure Zvar4 which was used as a reference.

Table 3. Tetrameric ligands, evaluated by Biacore (1M NaOH).

Ligand	Sequence	Remaining capacity 100 cycles (%)	Capacity relative to ref. 100 cycles
Zvar4	SEQ ID NO 21	27	1
Zvar(Q9A,N11E,N28A,N43A)4	SEQ ID NO 18	55	2.04
Zvar(Q9A,N11E,Q40V,A42K, N43E,L44I)4	SEQ ID NO 19	54	2.00
Zvar(Q9A,N11E,Q40V,A42K, N43A,L44I)4	SEQ ID NO 20	56	2.07

Example 4

[0075] The purified tetrameric ligands of Table 2 (all with an additional N-terminal cysteine) were immobilized on agarose beads using the methods described below and assessed for capacity and stability. The results are shown in Table 4 and Fig. 3.

Table 4. Matrices with tetrametric ligands, evaluated in columns (0.5 M NaOH).

Ligand	SEQ ID NO.	Ligand content (mg/ml)	Initial IgG capacity Qb10 (mg/ml)	Remaining IgG capacity Qb10 after six 4 h cycles (mg/ml)	Remaining IgG capacity after six 4 h cycles (%)	Capacity retention relative to ref. after six 4 h cycles
Zvar4	21	7	52.5	36.5	60	1
Zvar4	21	12	61.1	43.4	71	1
Zvar(Q9A, N11E,N28A, N43A)4	18	7.0	49.1	44.1	90	1.50
Zvar(Q9A, N11E,N28A, N43A)4	18	12.1	50.0	46.2	93	1.31

(continued)

Ligand	SEQ ID NO.	Ligand content (mg/ml)	Initial IgG capacity Qb10 (mg/ml)	Remaining IgG capacity Qb10 after six 4 h cycles (mg/ml)	Remaining IgG capacity after six 4 h cycles (%)	Capacity retention relative to ref. after six 4 h cycles
Zvar(Q9A, N11E,Q40V, A42K,N43A, L44I)4	20	7.2	49.0	44.2	90	1.50
Zvar(Q9A, N11E,Q40V, A42K,N43A, L44I)4	20	12.8	56.3	53.6	95	1.34
Zvar(N11K, H18K,S33K, D37E,A42R, N43A ,L44I, K50R,L51Y)4	30	9.7	56.3	52.0	92	1.53
Zvar(Q9A, N11K,H18K, D37E,A42R)4	31	10.8	56.9	52.5	92	1.30

Activation

[0076] The base matrix used was rigid cross-linked agarose beads of 85 micrometers (volume-weighted, d50V) median diameter, prepared according to the methods of US6602990 and with a pore size corresponding to an inverse gel filtration chromatography Kav value of 0.70 for dextran of Mw 110 kDa, according to the methods described in Gel Filtration Principles and Methods, Pharmacia LKB Biotechnology 1991, pp 6-13.

[0077] 25 mL (g) of drained base matrix, 10.0 mL distilled water and 2.02 g NaOH (s) was mixed in a 100 mL flask with mechanical stirring for 10 min at 25°C. 4.0 mL of epichlorohydrin was added and the reaction progressed for 2 hours. The activated gel was washed with 10 gel sediment volumes (GV) of water.

Coupling

[0078] To 20 mL of ligand solution (50 mg/mL) in a 50 ml Falcon tube, 169 mg $NaHCO_3$, 21 mg Na_2CO_3, 175 mg NaCl and 7 mg EDTA, was added. The Falcon tube was placed on a roller table for 5-10 min, and then 77 mg of DTE was added. Reduction proceeded for >45 min. The ligand solution was then desalted on a PD10 column packed with Sephadex G-25. The ligand content in the desalted solution was determined by measuring the 276 nm UV absorption.

[0079] The activated gel was washed with 3-5 GV {0.1 M phosphate/l mM EDTA pH 8.6} and the ligand was then coupled according to the method described in US6399750. All buffers used in the experiments had been degassed by nitrogen gas for at least 5-10 min. The ligand content of the gels could be controlled by varying the amount and concentration of the ligand solution.

[0080] After immobilization the gels were washed 3xGV with distilled water. The gels + 1 GV {0.1 M phosphate/l mM EDTA/10% thioglycerol pH 8.6} was mixed and the tubes were left in a shaking table at room temperature overnight. The gels were then washed alternately with 3xGV {0.1 M TRIS/0.15 M NaCl pH 8.6} and 0.5 M HAc and then 8-10xGV with distilled water. Gel samples were sent to an external laboratory for amino acid analysis and the ligand content (mg/ml gel) was calculated from the total amino acid content.

Protein

[0081] Gammanorm 165 mg/ml (Octapharma), diluted to 2mg/ml in Equilibration buffer.

Equilibration buffer

[0082] PBS Phosphate buffer 10 mM + 0.14 M NaCl + 0.0027 M KCl, pH 7,4 (Medicago)

Adsorption buffer

[0083] PBS Phosphate buffer 10 mM + 0.14 M NaCl + 0.0027 M KCl, pH 7,4 (Medicago)

Elution buffers

[0084] 100 mM acetate pH 2.9

Dynamic binding capacity

[0085] 2 ml of resin was packed in TRICORN™ 5 100 columns. The breakthrough capacity was determined with an ÄKTAExplorer 10 system at a residence time of 6 minutes (0.33 ml/min flow rate). Equilibration buffer was run through the bypass column until a stable baseline was obtained. This was done prior to auto zeroing. Sample was applied to the column until a 100% UV signal was obtained. Then, equilibration buffer was applied again until a stable baseline was obtained.

[0086] Sample was loaded onto the column until a UV signal of 85% of maximum absorbance was reached. The column was then washed with 5 column volumes (CV) equilibration buffer at flow rate 0.5ml/min. The protein was eluted with 5 CV elution buffer at a flow rate of 0.5 ml/min. Then the column was cleaned with 0.5M NaOH at flow rate 0.2 ml/min and reequilibrated with equilibration buffer.

[0087] For calculation of breakthrough capacity at 10%, the equation below was used. That is the amount of IgG that is loaded onto the column until the concentration of IgG in the column effluent is 10% of the IgG concentration in the feed.

$$q_{10\%} = \frac{C_0}{V_C}\left[V_{app} - V_{sys} - \int_{V_{sys}}^{V_{app}} \frac{A(V) - A_{sub}}{A_{100\%} - A_{sub}} * dv\right]$$

$A_{100\%}$ = 100% UV signal;
A_{sub} = absorbance contribution from non-binding IgG subclass;
$A(V)$ = absorbance at a given applied volume;
V_c = column volume;
V_{app} = volume applied until 10% breakthrough;
V_{sys} = system dead volume;
Co = feed concentration.

The dynamic binding capacity (DBC) at 10% breakthrough was calculated. The dynamic binding capacity (DBC) was calculated for 10 and 80% breakthrough.

CIP - 0.5 M NaOH

[0088] The 10% breakthrough DBC (Qb 10) was determined both before and after repeated exposures to alkaline cleaning solutions. Each cycle included a CIP step with 0.5 M NaOH pumped through the column at a rate of 0.5/min for 20 min, after which the column was left standing for 4 h. The exposure took place at room temperature (22 +/- 2°C). After this incubation, the column was washed with equilibration buffer for 20 min at a flow rate of 0.5 ml/min. Table 4 shows the remaining capacity after six 4 h cycles (i.e. 24 h cumulative exposure time to 0.5 M NaOH), both in absolute numbers and relative to the initial capacity.

<u>Example 5</u>

[0089] Example 4 was repeated with the tetrameric ligands shown in Table 5, but with 1.0 M NaOH used in the CIP steps instead of 0.5 M. The results are shown in Table 5 and in Fig. 4.

Table 5. Matrices with tetrametric ligands, evaluated in columns - 1.0 M NaOH.

Ligand	SEQ ID NO.	Ligand content (mg/ml)	Initial IgG capacity Qb10 (mg/ml)	Remaining IgG capacity Qb10 after six 4 h cycles (mg/ml)	Remaining IgG capacity after six 4 h cycles (%)	Capacity retention relative to ref. after six 4 h cycles
Zvar4	21	12	60.1	33.5	56	1
Zvar(Q9A, N11E,Q40V, A42K,N43A, L44I)4	20	12.8	60.3	56.0	93	1.67
Zvar(N11K, H18K,S33K, D37E,A42R, N43A ,L44I, K50R,L51Y)4	30	9.7	62.1	48.1	77	1.44

[0090] This written description uses examples to disclose the invention, including the best mode, and also to enable any person skilled in the art to practice the invention, including making and using any devices or systems and performing any incorporated methods. The patentable scope of the invention is defined by the claims, and may include other examples that occur to those skilled in the art. Such other examples are intended to be within the scope of the claims if they have structural elements that do not differ from the literal language of the claims, or if they include equivalent structural elements with insubstantial differences from the literal languages of the claims. All patents and patent applications mentioned in the text are hereby incorporated by reference in their entireties, as if they were individually incorporated.

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            20                  25                  30

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Lys Asp Asp Pro Ser Gln Ser Ala Ala Leu Leu Ala Glu Ala Lys Lys
            100                 105                 110

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        115                 120                 125

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Leu Pro Asn Leu Thr Glu Glu Gln Arg Asn Ala Phe Ile Gln Ser Leu
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Lys Asp Asp Pro Ser Val Ser Lys Glu Ile Leu Ala Glu Ala Lys Lys
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145   150   155   160

Ser Lys Glu Ile Leu Ala Glu Ala Lys Lys Leu Asn Asp Ala Gln Ala
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Pro Lys Val Asp Ala Lys Phe Asp Lys Glu Ala Gln Glu Ala Phe Tyr
            180   185   190

Glu Ile Leu His Leu Pro Asn Leu Thr Glu Glu Gln Arg Asn Ala Phe
        195   200   205

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1   5   10   15

Phe Tyr Glu Ile Leu His Leu Pro Asn Leu Thr Glu Glu Gln Arg Asn
            20   25   30

Ala Phe Ile Gln Ser Leu Lys Asp Asp Pro Ser Val Ser Lys Ala Ile
        35   40   45

Leu Ala Glu Ala Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys Val Asp
    50   55   60

Ala Lys Phe Asp Lys Glu Ala Gln Glu Ala Phe Tyr Glu Ile Leu His
65   70   75   80

Leu Pro Asn Leu Thr Glu Glu Gln Arg Asn Ala Phe Ile Gln Ser Leu
                85   90   95

Lys Asp Asp Pro Ser Val Ser Lys Ala Ile Leu Ala Glu Ala Lys Lys
            100   105   110

Leu Asn Asp Ala Gln Ala Pro Lys Val Asp Ala Lys Phe Asp Lys Glu
        115   120   125

Ala Gln Glu Ala Phe Tyr Glu Ile Leu His Leu Pro Asn Leu Thr Glu
    130   135   140

Glu Gln Arg Asn Ala Phe Ile Gln Ser Leu Lys Asp Asp Pro Ser Val
145   150   155   160

Ser Lys Ala Ile Leu Ala Glu Ala Lys Lys Leu Asn Asp Ala Gln Ala
                165   170   175

Pro Lys Val Asp Ala Lys Phe Asp Lys Glu Ala Gln Glu Ala Phe Tyr
180 185 190

Glu Ile Leu His Leu Pro Asn Leu Thr Glu Glu Gln Arg Asn Ala Phe
195 200 205

Ile Gln Ser Leu Lys Asp Asp Pro Ser Val Ser Lys Ala Ile Leu Ala
210 215 220

Glu Ala Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys Cys
225 230 235

<210> 21
<211> 237
<212> PRT
<213> Escherichia coli

<400> 21

Ala Gln Gly Thr Val Asp Ala Lys Phe Asp Lys Glu Gln Gln Asn Ala
1 5 10 15

Phe Tyr Glu Ile Leu His Leu Pro Asn Leu Thr Glu Glu Gln Arg Asn
20 25 30

Ala Phe Ile Gln Ser Leu Lys Asp Asp Pro Ser Gln Ser Ala Asn Leu
35 40 45

Leu Ala Glu Ala Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys Val Asp
50 55 60

Ala Lys Phe Asp Lys Glu Gln Gln Asn Ala Phe Tyr Glu Ile Leu His
65 70 75 80

Leu Pro Asn Leu Thr Glu Glu Gln Arg Asn Ala Phe Ile Gln Ser Leu
85 90 95

Lys Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala Lys Lys
100 105 110

Leu Asn Asp Ala Gln Ala Pro Lys Val Asp Ala Lys Phe Asp Lys Glu
115 120 125

Gln Gln Asn Ala Phe Tyr Glu Ile Leu His Leu Pro Asn Leu Thr Glu
130 135 140

Glu Gln Arg Asn Ala Phe Ile Gln Ser Leu Lys Asp Asp Pro Ser Gln
145 150 155 160

Ser Ala Asn Leu Leu Ala Glu Ala Lys Lys Leu Asn Asp Ala Gln Ala
165 170 175

Pro Lys Val Asp Ala Lys Phe Asp Lys Glu Gln Gln Asn Ala Phe Tyr
180 185 190

Glu Ile Leu His Leu Pro Asn Leu Thr Glu Glu Gln Arg Asn Ala Phe
195 200 205

Ile Gln Ser Leu Lys Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala
210 215 220

Glu Ala Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys Cys
225 230 235

<210> 22
<211> 58
<212> PRT
<213> Staphylococcus aureus

<400> 22

Ala Asp Asn Asn Phe Asn Lys Glu Gln Gln Asn Ala Phe Tyr Glu Ile
1 5 10 15

Leu Asn Met Pro Asn Leu Asn Glu Glu Gln Arg Asn Gly Phe Ile Gln
20 25 30

Ser Leu Lys Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ser Glu Ala
35 40 45

Lys Lys Leu Asn Glu Ser Gln Ala Pro Lys
50 55

<210> 23
<211> 58
<212> PRT
<213> Escherichia coli

<400> 23

Val Asp Ala Lys Phe Asp Lys Glu Gln Gln Lys Ala Phe Tyr Glu Ile
1 5 10 15

Leu Lys Leu Pro Asn Leu Thr Glu Glu Gln Arg Asn Ala Phe Ile Gln
20 25 30

Lys Leu Lys Asp Glu Pro Ser Gln Ser Arg Ala Ile Leu Ala Glu Ala
35 40 45

Lys Arg Tyr Asn Asp Ala Gln Ala Pro Lys
    50   55

<210> 24
<211> 58
<212> PRT
<213> Escherichia coli

<400> 24

Val Asp Ala Lys Phe Asp Lys Glu Ala Gln Glu Ala Phe Tyr Glu Ile
1   5   10   15

Leu His Leu Pro Asn Leu Thr Glu Glu Gln Arg Ala Ala Phe Ile Gln
            20   25   30

Ser Leu Lys Asp Asp Pro Ser Val Ser Lys Ala Ile Leu Ala Glu Ala
        35   40   45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
    50   55

<210> 25
<211> 58
<212> PRT
<213> Escherichia coli

<400> 25

Val Asp Ala Lys Phe Asp Lys Glu Ala Gln Lys Ala Phe Tyr Glu Ile
1   5   10   15

Leu Lys Leu Pro Asn Leu Thr Glu Glu Gln Arg Ala Ala Phe Ile Gln
            20   25   30

Lys Leu Lys Asp Glu Pro Ser Gln Ser Arg Ala Ile Leu Ala Glu Ala
        35   40   45

Lys Arg Tyr Asn Asp Ala Gln Ala Pro Lys
    50   55

<210> 26
<211> 58
<212> PRT
<213> Escherichia coli

<400> 26

Val Asp Ala Lys Phe Asp Lys Glu Gln Gln Lys Ala Phe Tyr Glu Ile
1   5   10   15

Leu Lys Leu Pro Asn Leu Thr Glu Glu Gln Arg Asn Ala Phe Ile Gln

20 25 30

Ser Leu Lys Asp Glu Pro Ser Gln Ser Arg Ala Ile Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

<210> 27
<211> 58
<212> PRT
<213> Escherichia coli

<400> 27

Val Asp Ala Lys Phe Asp Lys Glu Ala Gln Lys Ala Phe Tyr Glu Ile
1 5 10 15

Leu Lys Leu Pro Asn Leu Thr Glu Glu Gln Arg Asn Ala Phe Ile Gln
20 25 30

Ser Leu Lys Asp Glu Pro Ser Gln Ser Arg Ala Ile Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

<210> 28
<211> 58
<212> PRT
<213> Escherichia coli

<400> 28

Val Asp Ala Lys Phe Asp Lys Glu Ala Gln Lys Ala Phe Tyr Glu Ile
1 5 10 15

Leu Lys Leu Pro Asn Leu Thr Glu Glu Gln Arg Asn Ala Phe Ile Gln
20 25 30

Ser Leu Lys Asp Glu Pro Ser Gln Ser Arg Ala Ile Leu Ala Glu Ala
35 40 45

Lys Arg Leu Asn Asp Ala Gln Ala Pro Lys
50 55

<210> 29
<211> 58
<212> PRT
<213> Escherichia coli

<400> 29

Val Asp Ala Lys Phe Asp Lys Glu Ala Gln Lys Ala Phe Tyr Glu Ile
1   5   10   15

Leu Lys Leu Pro Asn Leu Thr Glu Glu Gln Arg Asn Ala Phe Ile Gln
            20   25   30

Ser Leu Lys Asp Glu Pro Ser Gln Ser Arg Asn Leu Leu Ala Glu Ala
        35   40   45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
    50   55

<210> 30
<211> 237
<212> PRT
<213> Escherichia coli

<400> 30

Ala Gln Gly Thr Val Asp Ala Lys Phe Asp Lys Glu Gln Gln Lys Ala
1   5   10   15

Phe Tyr Glu Ile Leu Lys Leu Pro Asn Leu Thr Glu Glu Gln Arg Asn
            20   25   30

Ala Phe Ile Gln Lys Leu Lys Asp Glu Pro Ser Gln Ser Arg Ala Ile
        35   40   45

Leu Ala Glu Ala Lys Arg Tyr Asn Asp Ala Gln Ala Pro Lys Val Asp
    50   55   60

Ala Lys Phe Asp Lys Glu Gln Gln Lys Ala Phe Tyr Glu Ile Leu Lys
65   70   75   80

Leu Pro Asn Leu Thr Glu Glu Gln Arg Asn Ala Phe Ile Gln Lys Leu
                85   90   95

Lys Asp Glu Pro Ser Gln Ser Arg Ala Ile Leu Ala Glu Ala Lys Arg
            100   105   110

Tyr Asn Asp Ala Gln Ala Pro Lys Val Asp Ala Lys Phe Asp Lys Glu
        115   120   125

Gln Gln Lys Ala Phe Tyr Glu Ile Leu Lys Leu Pro Asn Leu Thr Glu
    130   135   140

Glu Gln Arg Asn Ala Phe Ile Gln Lys Leu Lys Asp Glu Pro Ser Gln
145   150   155   160

Ser Arg Ala Ile Leu Ala Glu Ala Lys Arg Tyr Asn Asp Ala Gln Ala
165 170 175

Pro Lys Val Asp Ala Lys Phe Asp Lys Glu Gln Gln Lys Ala Phe Tyr
180 185 190

Glu Ile Leu Lys Leu Pro Asn Leu Thr Glu Glu Gln Arg Asn Ala Phe
195 200 205

Ile Gln Lys Leu Lys Asp Glu Pro Ser Gln Ser Arg Ala Ile Leu Ala
210 215 220

Glu Ala Lys Arg Tyr Asn Asp Ala Gln Ala Pro Lys Cys
225 230 235

<210> 31
<211> 237
<212> PRT
<213> Escherichia coli

<400> 31

Ala Gln Gly Thr Val Asp Ala Lys Phe Asp Lys Glu Ala Gln Lys Ala
1 5 10 15

Phe Tyr Glu Ile Leu Lys Leu Pro Asn Leu Thr Glu Glu Gln Arg Asn
20 25 30

Ala Phe Ile Gln Ser Leu Lys Asp Glu Pro Ser Gln Ser Arg Asn Leu
35 40 45

Leu Ala Glu Ala Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys Val Asp
50 55 60

Ala Lys Phe Asp Lys Glu Ala Gln Lys Ala Phe Tyr Glu Ile Leu Lys
65 70 75 80

Leu Pro Asn Leu Thr Glu Glu Gln Arg Asn Ala Phe Ile Gln Ser Leu
85 90 95

Lys Asp Glu Pro Ser Gln Ser Arg Asn Leu Leu Ala Glu Ala Lys Lys
100 105 110

Leu Asn Asp Ala Gln Ala Pro Lys Val Asp Ala Lys Phe Asp Lys Glu
115 120 125

Ala Gln Lys Ala Phe Tyr Glu Ile Leu Lys Leu Pro Asn Leu Thr Glu
130 135 140

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Glu Gln Arg Asn Ala Phe Ile Gln Ser Leu Lys Asp Glu Pro Ser Gln
145                 150                 155                 160

Ser Arg Asn Leu Leu Ala Glu Ala Lys Lys Leu Asn Asp Ala Gln Ala
                165                 170                 175

Pro Lys Val Asp Ala Lys Phe Asp Lys Glu Ala Gln Lys Ala Phe Tyr
            180                 185                 190

Glu Ile Leu Lys Leu Pro Asn Leu Thr Glu Glu Gln Arg Asn Ala Phe
        195                 200                 205

Ile Gln Ser Leu Lys Asp Glu Pro Ser Gln Ser Arg Asn Leu Leu Ala
    210                 215                 220

Glu Ala Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys Cys
225                 230                 235


<210>  32
<211>  237
<212>  PRT
<213>  Escherichia coli

<400>  32

Ala Gln Gly Thr Val Asp Ala Lys Phe Asp Lys Glu Ala Gln Glu Ala
1               5                   10                  15

Phe Tyr Glu Ile Leu His Leu Pro Asn Leu Thr Glu Glu Gln Arg Ala
            20                  25                  30

Ala Phe Ile Gln Ser Leu Lys Asp Asp Pro Ser Val Ser Lys Ala Ile
        35                  40                  45

Leu Ala Glu Ala Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys Val Asp
    50                  55                  60

Ala Lys Phe Asp Lys Glu Ala Gln Glu Ala Phe Tyr Glu Ile Leu His
65                  70                  75                  80

Leu Pro Asn Leu Thr Glu Glu Gln Arg Ala Ala Phe Ile Gln Ser Leu
                85                  90                  95

Lys Asp Asp Pro Ser Val Ser Lys Ala Ile Leu Ala Glu Ala Lys Lys
            100                 105                 110

Leu Asn Asp Ala Gln Ala Pro Lys Val Asp Ala Lys Phe Asp Lys Glu
        115                 120                 125
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Ala Gln Glu Ala Phe Tyr Glu Ile Leu His Leu Pro Asn Leu Thr Glu
    130   135   140

Glu Gln Arg Ala Ala Phe Ile Gln Ser Leu Lys Asp Asp Pro Ser Val
145   150   155   160

Ser Lys Ala Ile Leu Ala Glu Ala Lys Lys Leu Asn Asp Ala Gln Ala
                165   170   175

Pro Lys Val Asp Ala Lys Phe Asp Lys Glu Ala Gln Glu Ala Phe Tyr
            180   185   190

Glu Ile Leu His Leu Pro Asn Leu Thr Glu Glu Gln Arg Ala Ala Phe
        195   200   205

Ile Gln Ser Leu Lys Asp Asp Pro Ser Val Ser Lys Ala Ile Leu Ala
    210   215   220

Glu Ala Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys Cys
225   230   235

<210> 33
<211> 353
<212> PRT
<213> Escherichia coli

<400> 33

Ala Gln Gly Thr Val Asp Ala Lys Phe Asp Lys Glu Ala Gln Glu Ala
1   5   10   15

Phe Tyr Glu Ile Leu His Leu Pro Asn Leu Thr Glu Glu Gln Arg Asn
            20   25   30

Ala Phe Ile Gln Ser Leu Lys Asp Asp Pro Ser Val Ser Lys Ala Ile
        35   40   45

Leu Ala Glu Ala Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys Val Asp
    50   55   60

Ala Lys Phe Asp Lys Glu Ala Gln Glu Ala Phe Tyr Glu Ile Leu His
65   70   75   80

Leu Pro Asn Leu Thr Glu Glu Gln Arg Asn Ala Phe Ile Gln Ser Leu
                85   90   95

Lys Asp Asp Pro Ser Val Ser Lys Ala Ile Leu Ala Glu Ala Lys Lys
            100   105   110

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Leu Asn Asp Ala Gln Ala Pro Lys Val Asp Ala Lys Phe Asp Lys Glu
        115                 120                 125

Ala Gln Glu Ala Phe Tyr Glu Ile Leu His Leu Pro Asn Leu Thr Glu
    130                 135                 140

Glu Gln Arg Asn Ala Phe Ile Gln Ser Leu Lys Asp Asp Pro Ser Val
145                 150                 155                 160

Ser Lys Ala Ile Leu Ala Glu Ala Lys Lys Leu Asn Asp Ala Gln Ala
                165                 170                 175

Pro Lys Val Asp Ala Lys Phe Asp Lys Glu Ala Gln Glu Ala Phe Tyr
            180                 185                 190

Glu Ile Leu His Leu Pro Asn Leu Thr Glu Glu Gln Arg Asn Ala Phe
        195                 200                 205

Ile Gln Ser Leu Lys Asp Asp Pro Ser Val Ser Lys Ala Ile Leu Ala
    210                 215                 220

Glu Ala Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys Val Asp Ala Lys
225                 230                 235                 240

Phe Asp Lys Glu Ala Gln Glu Ala Phe Tyr Glu Ile Leu His Leu Pro
                245                 250                 255

Asn Leu Thr Glu Glu Gln Arg Asn Ala Phe Ile Gln Ser Leu Lys Asp
            260                 265                 270

Asp Pro Ser Val Ser Lys Ala Ile Leu Ala Glu Ala Lys Lys Leu Asn
        275                 280                 285

Asp Ala Gln Ala Pro Lys Val Asp Ala Lys Phe Asp Lys Glu Ala Gln
    290                 295                 300

Glu Ala Phe Tyr Glu Ile Leu His Leu Pro Asn Leu Thr Glu Glu Gln
305                 310                 315                 320

Arg Asn Ala Phe Ile Gln Ser Leu Lys Asp Asp Pro Ser Val Ser Lys
                325                 330                 335

Ala Ile Leu Ala Glu Ala Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
            340                 345                 350

Cys
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<210> 34
<211> 237
<212> PRT
<213> Escherichia coli

<400> 34

Ala Gln Gly Thr Val Asp Ala Lys Phe Asp Lys Glu Ala Gln Glu Ala
1 5 10 15

Phe Tyr Glu Ile Leu His Leu Pro Asn Leu Thr Glu Glu Gln Arg Asn
20 25 30

Ala Phe Ile Gln Ser Leu Lys Asp Glu Pro Ser Val Ser Lys Ala Ile
35 40 45

Leu Ala Glu Ala Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys Val Asp
50 55 60

Ala Lys Phe Asp Lys Glu Ala Gln Glu Ala Phe Tyr Glu Ile Leu His
65 70 75 80

Leu Pro Asn Leu Thr Glu Glu Gln Arg Asn Ala Phe Ile Gln Ser Leu
85 90 95

Lys Asp Glu Pro Ser Val Ser Lys Ala Ile Leu Ala Glu Ala Lys Lys
100 105 110

Leu Asn Asp Ala Gln Ala Pro Lys Val Asp Ala Lys Phe Asp Lys Glu
115 120 125

Ala Gln Glu Ala Phe Tyr Glu Ile Leu His Leu Pro Asn Leu Thr Glu
130 135 140

Glu Gln Arg Asn Ala Phe Ile Gln Ser Leu Lys Asp Glu Pro Ser Val
145 150 155 160

Ser Lys Ala Ile Leu Ala Glu Ala Lys Lys Leu Asn Asp Ala Gln Ala
165 170 175

Pro Lys Val Asp Ala Lys Phe Asp Lys Glu Ala Gln Glu Ala Phe Tyr
180 185 190

Glu Ile Leu His Leu Pro Asn Leu Thr Glu Glu Gln Arg Asn Ala Phe
195 200 205

Ile Gln Ser Leu Lys Asp Glu Pro Ser Val Ser Lys Ala Ile Leu Ala
210 215 220

Glu Ala Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys Cys
225 230 235

<210> 35
<211> 237
<212> PRT
<213> Escherichia coli

<400> 35

Ala Gln Gly Thr Val Asp Ala Lys Phe Asp Lys Glu Ala Gln Glu Ala
1 5 10 15

Phe Tyr Glu Ile Leu His Leu Pro Asn Leu Thr Glu Glu Gln Arg Asn
20 25 30

Ala Phe Ile Gln Ser Leu Lys Asp Glu Pro Ser Val Ser Arg Ala Ile
35 40 45

Leu Ala Glu Ala Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys Val Asp
50 55 60

Ala Lys Phe Asp Lys Glu Ala Gln Glu Ala Phe Tyr Glu Ile Leu His
65 70 75 80

Leu Pro Asn Leu Thr Glu Glu Gln Arg Asn Ala Phe Ile Gln Ser Leu
85 90 95

Lys Asp Glu Pro Ser Val Ser Arg Ala Ile Leu Ala Glu Ala Lys Lys
100 105 110

Leu Asn Asp Ala Gln Ala Pro Lys Val Asp Ala Lys Phe Asp Lys Glu
115 120 125

Ala Gln Glu Ala Phe Tyr Glu Ile Leu His Leu Pro Asn Leu Thr Glu
130 135 140

Glu Gln Arg Asn Ala Phe Ile Gln Ser Leu Lys Asp Glu Pro Ser Val
145 150 155 160

Ser Arg Ala Ile Leu Ala Glu Ala Lys Lys Leu Asn Asp Ala Gln Ala
165 170 175

Pro Lys Val Asp Ala Lys Phe Asp Lys Glu Ala Gln Glu Ala Phe Tyr
180 185 190

Glu Ile Leu His Leu Pro Asn Leu Thr Glu Glu Gln Arg Asn Ala Phe
195 200 205

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Ile Gln Ser Leu Lys Asp Glu Pro Ser Val Ser Arg Ala Ile Leu Ala
    210                 215                 220


Glu Ala Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys Cys
225                 230                 235


<210>  36
<211>  58
<212>  PRT
<213>  Escherichia coli

<400>  36

Ala Asp Asn Lys Phe Asn Lys Glu Ala Gln Glu Ala Phe Tyr Glu Ile
1               5                   10                  15


Leu His Leu Pro Asn Leu Asn Glu Glu Gln Arg Asn Gly Phe Ile Gln
            20                  25                  30


Ser Leu Lys Asp Asp Pro Ser Val Ser Lys Ala Ile Leu Ala Glu Ala
        35                  40                  45


Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
    50                  55


<210>  37
<211>  58
<212>  PRT
<213>  Escherichia coli

<400>  37

Ala Asp Asn Lys Phe Asn Lys Glu Ala Gln Glu Ala Phe Tyr Glu Ile
1               5                   10                  15


Leu His Leu Pro Asn Leu Thr Glu Glu Gln Arg Asn Gly Phe Ile Gln
            20                  25                  30


Ser Leu Lys Asp Asp Pro Ser Val Ser Lys Ala Ile Leu Ala Glu Ala
        35                  40                  45


Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
    50                  55


<210>  38
<211>  58
<212>  PRT
<213>  Escherichia coli

<400>  38
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Val Asp Ala Lys Phe Asp Lys Glu Gln Gln Tyr Ala Phe Tyr Glu Ile
1               5                   10                  15

Leu His Leu Pro Asn Leu Thr Glu Glu Gln Arg Asn Ala Phe Ile Gln
            20                  25                  30

Ser Leu Lys Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
        35                  40                  45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
    50                  55


<210>  39
<211>  58
<212>  PRT
<213>  Escherichia coli

<400>  39

Val Asp Ala Lys Phe Asp Lys Glu Gln Gln Thr Ala Phe Tyr Glu Ile
1               5                   10                  15

Leu His Leu Pro Asn Leu Thr Glu Glu Gln Arg Asn Ala Phe Ile Gln
            20                  25                  30

Ser Leu Lys Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
        35                  40                  45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
    50                  55


<210>  40
<211>  58
<212>  PRT
<213>  Escherichia coli

<400>  40

Val Asp Ala Lys Phe Asp Lys Glu Gln Gln Phe Ala Phe Tyr Glu Ile
1               5                   10                  15

Leu His Leu Pro Asn Leu Thr Glu Glu Gln Arg Asn Ala Phe Ile Gln
            20                  25                  30

Ser Leu Lys Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
        35                  40                  45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
    50                  55


<210>  41
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<211> 58
<212> PRT
<213> Escherichia coli

<400> 41

Val Asp Ala Lys Phe Asp Lys Glu Gln Gln Leu Ala Phe Tyr Glu Ile
1   5   10   15

Leu His Leu Pro Asn Leu Thr Glu Glu Gln Arg Asn Ala Phe Ile Gln
            20   25   30

Ser Leu Lys Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
        35   40   45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
    50   55

<210> 42
<211> 58
<212> PRT
<213> Escherichia coli

<400> 42

Val Asp Ala Lys Phe Asp Lys Glu Gln Gln Trp Ala Phe Tyr Glu Ile
1   5   10   15

Leu His Leu Pro Asn Leu Thr Glu Glu Gln Arg Asn Ala Phe Ile Gln
            20   25   30

Ser Leu Lys Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
        35   40   45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
    50   55

<210> 43
<211> 58
<212> PRT
<213> Escherichia coli

<400> 43

Val Asp Ala Lys Phe Asp Lys Glu Gln Gln Ile Ala Phe Tyr Glu Ile
1   5   10   15

Leu His Leu Pro Asn Leu Thr Glu Glu Gln Arg Asn Ala Phe Ile Gln
            20   25   30

Ser Leu Lys Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
        35   40   45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

<210> 44
<211> 58
<212> PRT
<213> Escherichia coli

<400> 44

Val Asp Ala Lys Phe Asp Lys Glu Gln Gln Met Ala Phe Tyr Glu Ile
1 5 10 15

Leu His Leu Pro Asn Leu Thr Glu Glu Gln Arg Asn Ala Phe Ile Gln
20 25 30

Ser Leu Lys Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

<210> 45
<211> 58
<212> PRT
<213> Escherichia coli

<400> 45

Val Asp Ala Lys Phe Asp Lys Glu Gln Gln Val Ala Phe Tyr Glu Ile
1 5 10 15

Leu His Leu Pro Asn Leu Thr Glu Glu Gln Arg Asn Ala Phe Ile Gln
20 25 30

Ser Leu Lys Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

<210> 46
<211> 58
<212> PRT
<213> Escherichia coli

<400> 46

Val Asp Ala Lys Phe Asp Lys Glu Gln Gln Ala Ala Phe Tyr Glu Ile
1 5 10 15

Leu His Leu Pro Asn Leu Thr Glu Glu Gln Arg Asn Ala Phe Ile Gln

20 25 30

Ser Leu Lys Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

<210> 47
<211> 58
<212> PRT
<213> Escherichia coli

<400> 47

Val Asp Ala Lys Phe Asp Lys Glu Gln Gln His Ala Phe Tyr Glu Ile
1 5 10 15

Leu His Leu Pro Asn Leu Thr Glu Glu Gln Arg Asn Ala Phe Ile Gln
20 25 30

Ser Leu Lys Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

<210> 48
<211> 58
<212> PRT
<213> Escherichia coli

<400> 48

Val Asp Ala Lys Phe Asp Lys Glu Gln Gln Arg Ala Phe Tyr Glu Ile
1 5 10 15

Leu His Leu Pro Asn Leu Thr Glu Glu Gln Arg Asn Ala Phe Ile Gln
20 25 30

Ser Leu Lys Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

<210> 49
<211> 58
<212> PRT
<213> Escherichia coli

<400> 49

Val Asp Ala Lys Phe Asp Lys Glu Ala Gln Glu Ala Phe Tyr Glu Ile
1   5   10   15

Leu His Leu Pro Asn Leu Thr Glu Glu Gln Arg Asn Ala Phe Ile Gln
            20   25   30

Ser Leu Lys Asp Glu Pro Ser Val Ser Lys Ala Ile Leu Ala Glu Ala
        35   40   45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
    50   55

<210> 50
<211> 58
<212> PRT
<213> Escherichia coli

<400> 50

Val Asp Ala Lys Phe Asp Lys Glu Ala Gln Glu Ala Phe Tyr Glu Ile
1   5   10   15

Leu His Leu Pro Asn Leu Thr Glu Glu Gln Arg Asn Ala Phe Ile Gln
            20   25   30

Ser Leu Lys Asp Glu Pro Ser Val Ser Arg Ala Ile Leu Ala Glu Ala
        35   40   45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
    50   55

<210> 51
<211> 52
<212> PRT
<213> Escherichia coli

<400> 51

Lys Glu Gln Gln Asn Ala Phe Tyr Glu Ile Leu His Leu Pro Asn Leu
1   5   10   15

Thr Glu Glu Gln Arg Asn Ala Phe Ile Gln Ser Leu Lys Asp Asp Pro
            20   25   30

Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala Lys Lys Leu Asn Asp Ala
        35   40   45

Gln Ala Pro Lys
    50

<210> 52
<211> 52
<212> PRT
<213> Staphylococcus aureus

<400> 52

Lys Glu Gln Gln Asn Ala Phe Tyr Glu Ile Leu His Leu Pro Asn Leu
1 5 10 15

Thr Glu Glu Gln Arg Asn Gly Phe Ile Gln Ser Leu Lys Asp Asp Pro
20 25 30

Ser Val Ser Lys Glu Ile Leu Ala Glu Ala Lys Lys Leu Asn Asp Ala
35 40 45

Gln Ala Pro Lys
50

<210> 53
<211> 52
<212> PRT
<213> Escherichia coli

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<220>
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<223> Xaa can be any naturally occurring amino acid

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<220>
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<223>  Xaa can be any naturally occurring amino acid

<400>  53

Lys Glu Xaa Gln Xaa Ala Phe Tyr Glu Ile Leu Xaa Leu Pro Asn Leu
1               5                   10                  15

Thr Glu Glu Gln Arg Xaa Xaa Phe Ile Xaa Xaa Leu Lys Asp Xaa Pro
            20                  25                  30

Ser Xaa Ser Xaa Xaa Xaa Leu Ala Glu Ala Lys Xaa Xaa Asn Asp Ala
        35                  40                  45

Gln Ala Pro Lys
    50
```

Claims

1. An Fc-binding polypeptide comprising a mutant of an Fc-binding domain of Staphylococcus Protein A (SpA), as defined by, or having at least 90% such as at least 95% or 98% identity to, SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO:3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:22, SEQ ID NO 51 or SEQ ID NO 52, wherein at least the asparagine or serine residue at the position corresponding to position 11 in SEQ ID NO:4-7 has been mutated to an amino acid selected from the group consisting of glutamic acid, lysine, tyrosine, threonine, phenylalanine, leucine, isoleucine, tryptophan, methionine, valine, alanine, histidine and arginine.

2. The polypeptide of claim 1, comprising a mutant of a parental Fc-binding domain of Staphylococcus Protein A (SpA), as defined by, or having at least 90% such as at least 95% or 98% identity to, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO 51 or SEQ ID NO 52.

3. The polypeptide of claim 1 or 2, wherein:

   i) the amino acid residue at the position corresponding to position 11 in SEQ ID NO:4-7 is a glutamic acid;
   ii) the amino acid residue at the position corresponding to position 11 in SEQ ID NO:4-7 is a lysine;
   iii) the amino acid residue at the position corresponding to position 9 in SEQ ID NO:4-7 is an alanine;
   iv) the amino acid residue at the position corresponding to position 50 in SEQ ID NO:4-7 is an arginine or a glutamic acid, such as an arginine;
   v) the amino acid residue at the position corresponding to position 3 in SEQ ID NO:4-7 is an alanine and/or the amino acid residue at the position corresponding to position 6 in SEQ ID NO:4-7 is an aspartic acid;
   vi) at least one, such as both, of the amino acid residues at the positions corresponding to positions 3 and 6 in SEQ ID NO:4-7 is an asparagine;
   vii) the amino acid residue at the position corresponding to position 43 in SEQ ID NO:4-7 is an alanine or a glutamic acid, such as an alanine;
   viii) the amino acid residue at the position corresponding to position 28 in SEQ ID NO:4-7 is an alanine or an asparagine;
   ix) the amino acid residue at the position corresponding to position 40 in SEQ ID NO:4-7 is selected from the group consisting of asparagine, alanine, glutamic acid and valine;
   x) the amino acid residue at the position corresponding to position 42 in SEQ ID NO:4-7 is an alanine, lysine

or arginine, such as an arginine;
xi) the amino acid residue at the position corresponding to position 44 in SEQ ID NO:4-7 is a leucine or an isoleucine, such as an isoleucine;
xii) the amino acid residue at the position corresponding to position 18 in SEQ ID NO:4-7 is a lysine or a histidine, such as a lysine;
xiii) the amino acid residue at the position corresponding to position 33 in SEQ ID NO:4-7 is a lysine or a serine, such as a lysine;
xiv) the amino acid residue at the position corresponding to position 37 in SEQ ID NO:4-7 is a glutamic acid or an aspartic acid, such as a glutamic acid;
xv) the amino acid residue at the position corresponding to position 51 in SEQ ID NO:4-7 is a tyrosine or a leucine, such as a tyrosine; and/or
xvi) the amino acid residues at the positions corresponding to positions 1, 2, 3 and 4 or to positions 3, 4, 5 and 6 in SEQ ID NO: 4-7 have been deleted.

4. The polypeptide according to any preceding claim, which is a mutant of Zvar as defined by SEQ ID NO:7, wherein the amino acid residue at position 9 is alanine and the amino acid residue at position 11 is lysine or glutamic acid, such as lysine.

5. The polypeptide according to claim 4, wherein:

i) the amino acid residue at position 43 is alanine or glutamic acid;
ii) the amino acid residue at position 40 is valine; and/or
iii) the amino acid residue at position 44 is isoleucine.

6. The polypeptide according to any preceding claim, wherein the mutation is selected from:

i) the group consisting of: N11K; N11E; N11Y; N11T; N11F; N11L; N11W; N11I; N11M; N11V; N11A; N11H; N11R; N11E,Q32A; N11E,Q32E,Q40E; N11E,Q32E,K50R; Q9A,N11E,N43A; Q9A,N11E,N28A,N43A; Q9A,N11E,Q40V,A42K,N43E,L44I; Q9A,N11E,Q40V,A42K,N43A,L44I; N11K,H18K,S33K,D37E,A42R,N43A,L44I,K50R,L51Y; Q9A,N11E,N28A,Q40V,A42K,N43A,L44I; Q9A,N11K,H18K,S33K,D37E,A42R,N43A,L44I,K50R,L51Y; N11K, H18K, D37E, A42R, N43A, L44I; Q9A, N11K, H18K, D37E, A42R, N43A, L44I; Q9A, N11K, H18K, D37E, A42R, N43A, L44I, K50R; Q9A, N11K, H18K, D37E, A42R; Q9A, N11E, D37E, Q40V, A42K, N43A, L44I and Q9A, N11E, D37E, Q40V, A42R, N43 A, L44I; or
ii) the group consisting of: N11K; N11Y; N11F; N11L; N11W; N11I; N11M; N11V; N11A; N11H; N11R; Q9A, N11E, N43A; Q9A, N11E, N28A, N43A; Q9A, N11E, Q40V, A42K, N43E, L44I; Q9A, N11E, Q40V, A42K, N43A, L44I; Q9A, N11E, N28A, Q40V, A42K, N43A, L44I; N11K, H18K,S33K, D37E, A42R, N43A, L44I, K50R, L51Y; Q9A,N11K, H18K, S33K, D37E, A42R, N43A, L44I, K50R, L51Y; N11K, H18K, D37E, A42R, N43A, L44I; Q9A, N11K, H18K, D37E, A42R, N43A, L44I and Q9A, N11K, H18K, D37E, A42R, N43A, L44I, K50R.

7. The polypeptide according to any preceding claim, comprising or consisting essentially of a sequence selected from:

i) the group consisting of: SEQ ID NO 8, SEQ ID NO 9, SEQ ID NO 10, SEQ ID NO 11, SEQ ID NO 12, SEQ ID NO 13, SEQ ID NO 14, SEQ ID NO 15, SEQ ID NO 16, SEQ ID NO 23, SEQ ID NO 24, SEQ ID NO 25, SEQ ID NO 26, SEQ ID NO 27, SEQ ID NO 28, SEQ ID NO 29, SEQ ID NO 36, SEQ ID NO 37, SEQ ID NO 38, SEQ ID NO 39, SEQ ID NO 40, SEQ ID NO 41, SEQ ID NO 42, SEQ ID NO 43, SEQ ID NO 44, SEQ ID NO 45, SEQ ID NO 46, SEQ ID NO 47, SEQ ID NO 48, SEQ ID NO 49 and SEQ ID NO 50;
ii) the group consisting of: SEQ ID NO 8, SEQ ID NO 9, SEQ ID NO 10, SEQ ID NO 11, SEQ ID NO 16, SEQ ID NO 23, SEQ ID NO 24, SEQ ID NO 25, SEQ ID NO 26, SEQ ID NO 27, SEQ ID NO 28 and SEQ ID NO 29; and/or
iii) the group consisting of: SEQ ID NO 8, SEQ ID NO 9, SEQ ID NO 10, SEQ ID NO 11, SEQ ID NO 16, SEQ ID NO 23, SEQ ID NO 24, SEQ ID NO 25, SEQ ID NO 27, SEQ ID NO 28, SEQ ID NO 38, SEQ ID NO 40; SEQ ID NO 41; SEQ ID NO 42; SEQ NO 43, SEQ ID NO 44, SEQ ID NO 45, SEQ ID NO 46, SEQ ID NO 47 and SEQ ID NO 48.

8. The polypeptide according to any preceding claim, which polypeptide has an improved alkaline stability compared to a polypeptide or a parental polypeptide as defined by SEQ ID NO 1, SEQ ID NO 2, SEQ ID NO 3, SEQ ID NO 4, SEQ ID NO 5, SEQ ID NO 6 or SEQ ID NO 7, such as by SEQ ID NO 7, wherein optionally the alkaline stability is improved as measured by the remaining IgG-binding capacity after 24 or 25 h incubation in 0.5 M or 1.0 M aqueous NaOH at 22 +/- 2 °C.

9. An Fc-binding polypeptide which comprises a sequence as defined by, or having at least 90% or at least 95% or 98% identity to SEQ ID NO 53.

$KEX_1Q\ X_2AFYEILX_3LP\ NLTEEQRX_4X_5F\ IX_6X_7LKDX_8PSX_9\ SX_{10}X_{11}X_{12}LAEAKX_{13}\ X_{14}NDAQAPK$ (SEQ ID NO 53)

wherein individually of each other:

X_1=A or Q
X_2=E,K,Y,T,F,L,W,I,M,V,A,H or R
X_3=H or K
X_4=A or N
X_5=A or G
X_6=Q or E
X_7=S or K
X_8=E or D
X_9=Q or V
X_{10}=K,R or A
X_{11}=A,E or N
X_{12}=I or L
X_{13}=K or R
X_{14}=L or Y

10. A multimer comprising or consisting essentially of a plurality of polypeptides as defined by any preceding claim, wherein optionally:

i) the polypeptides are linked by linkers comprising up to 15 amino acids;
ii) the multimer is a dimer, trimer, tetramer, pentamer, hexamer, heptamer, octamer or nonamer; and/or
iii) the multimer comprises or consists essentially of a sequence selected from the group of sequences defined by SEQ ID NO 17, SEQ ID NO 18, SEQ ID NO 19, SEQ ID NO 20, SEQ ID NO 30, SEQ ID NO 31, SEQ ID NO 32, SEQ ID NO 33, SEQ ID NO 34 and SEQ ID NO 35.

11. The polypeptide or multimer according to any preceding claim, further comprising at, or within 1-5 amino acid residues from, the C-terminal or N-terminal one or more coupling element, selected from the group consisting of one or more cysteine residues, a plurality of lysine residues and a plurality of histidine residues.

12. A nucleic acid or a vector encoding a polypeptide or multimer according to any preceding claim, or an expression system, which comprises said nucleic acid or vector.

13. A separation matrix, wherein a plurality of polypeptides or multimers according to any one of claims 1 - 11 have been coupled to a solid support.

14. The separation matrix according to claim 13, wherein:

i) the polypeptides or multimers have been coupled to the solid support via thioether bonds.
ii) the solid support is a polysaccharide;
iii) the IgG capacity of the matrix after 24 incubation in 0.5 M NaOH at 22 +/- 2 °C is at least 80, such as at least 85, at least 90 or at least 95% of the IgG capacity before the incubation; and/or
iv) the IgG capacity of the matrix after 24 incubation in 1.0 M NaOH at 22 +/- 2 °C is at least 70, such as at least 80 or at least 90% of the IgG capacity before the incubation.

15. A method of isolating an immunoglobulin, wherein a separation matrix according to any one of claims 13-14 is used.

16. The method of claim 15, comprising the steps of:

a) contacting a liquid sample comprising an immunoglobulin with a separation matrix according to any one of claims 13-14,

b) washing said separation matrix with a washing liquid,
c) eluting the immunoglobulin from the separation matrix with an elution liquid, and
d) cleaning the separation matrix with a cleaning liquid.

17. The method of claim 16, wherein:

i) the cleaning liquid is alkaline, such as with a pH of 13 - 14;
ii) the cleaning liquid comprises 0.1 - 1.0 M NaOH or KOH, such as 0.5 - 1.0 M NaOH or KOH;
iii) steps a) - d) are repeated at least 10 times, such as at least 50 times or 50 - 200 times; and/or
iv) steps a) - c) are repeated at least 10 times, such as at least 50 times or 50 - 200 times and wherein step d) is performed after a plurality of instances of step c), such as at least 10 or at least 50 times.

Alignment of Fc-binding domains

```
E       --- -------AQQ  NAFYQVLNMP  NLNADQRNGF  IQSLKDDPSQ  SANVLGEAQK  LNDSQAPK  51  (SEQ ID NO: 1)
D       ADA QQNKFNKDQQ  SAFYEILNMP  NLNEEQRNGF  IQSLKDDPSQ  STNVLGEAKK  LNESQAPK  61  (SEQ ID NO: 2)
A       --A DNN-FNKEQQ  NAFYEILNMP  NLNEEQRNGF  IQSLKDDPSQ  SANLLAEAKK  LNESQAPK  58  (SEQ ID NO: 3)
B       --- ADNKFNKEQQ  NAFYEILHLP  NLNEEQRNGF  IQSLKDDPSQ  SANLLAEAKK  LNDAQAPK  58  (SEQ ID NO: 4)
C       --- ADNKFNKEQQ  NAFYEILHLP  NLTEEQRNGF  IQSLKDDPSV  SKEILAEAKK  LNDAQAPK  58  (SEQ ID NO: 5)
Z       --- VDNKFNKEQQ  NAFYEILHLP  NLNEEQRNAF  IQSLKDDPSQ  SANLLAEAKK  LNDAQAPK  58  (SEQ ID NO: 6)
Zvar    --- VDAKFDKEQQ  NAFYEILHLP  NLTEEQRNAF  IQSLKDDPSQ  SANLLAEAKK  LNDAQAPK  58  (SEQ ID NO: 7)

        --- ------KEQQ  NAFYEILHLP  NLTEEQRNAF  IQSLKDDPSQ  SANLLAEAKK  LNDAQAPK  52  (SEQ ID NO: 51)
        --- ------KEQQ  NAFYEILHLP  NLTEEQRNGF  IQSLKDDPSV  SKEILAEAKK  LNDAQAPK  52  (SEQ ID NO: 52)

Pos         1        10          20          30          40          50       58
```

Fig. 1

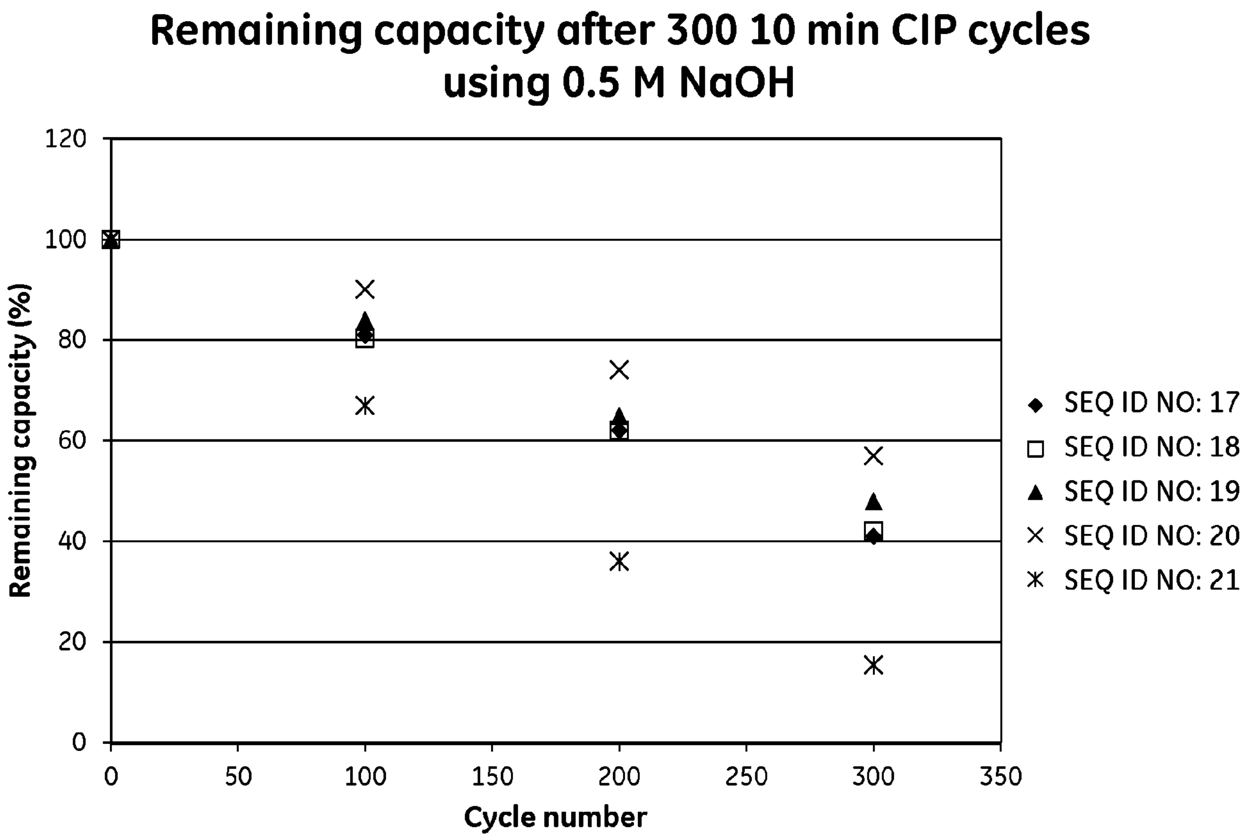
Remaining capacity after 300 10 min CIP cycles
using 0.5 M NaOH
Remaining capacity (%)
120
100
80
60
40
20
0
0
50
100
150
200
250
300
350
Cycle number
SEQ ID NO: 17
SEQ ID NO: 18
SEQ ID NO: 19
SEQ ID NO: 20
SEQ ID NO: 21

Fig. 2.

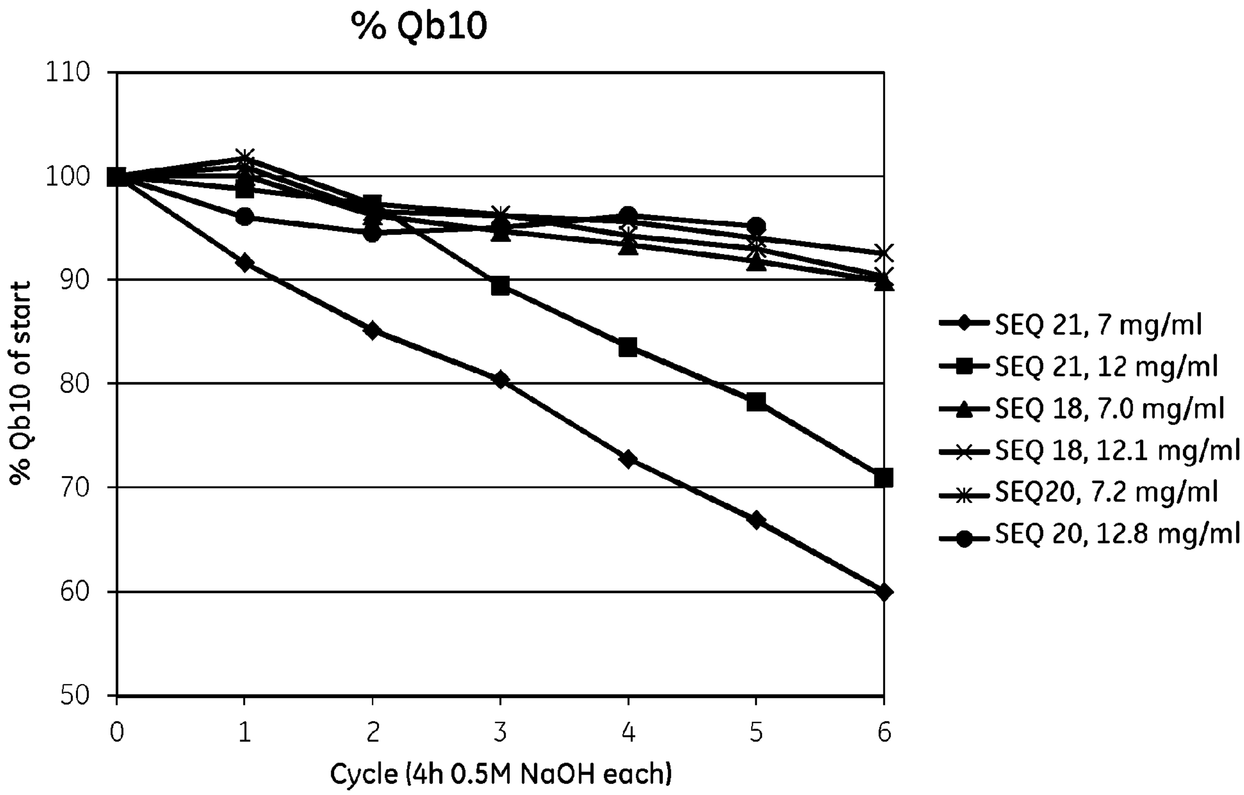
% Qb10
110
100
90
80
70
60
50
% Qb10 of start
0
1
2
3
4
5
6
Cycle (4h 0.5M NaOH each)
SEQ 21, 7 mg/ml
SEQ 21, 12 mg/ml
SEQ 18, 7.0 mg/ml
SEQ 18, 12.1 mg/ml
SEQ20, 7.2 mg/ml
SEQ 20, 12.8 mg/ml

Fig. 3.

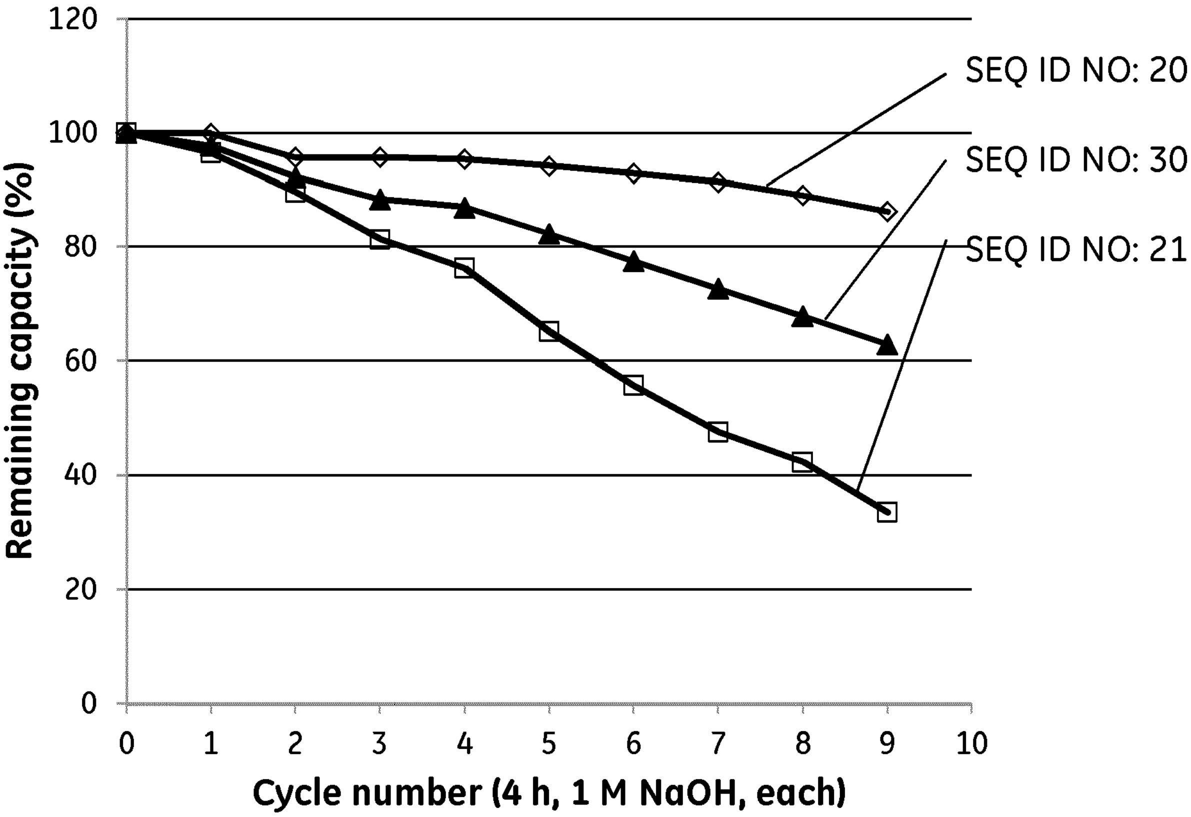
120
100
80
60
40
20
0
Remaining capacity (%)
0
1
2
3
4
5
6
7
8
9
10
Cycle number (4 h, 1 M NaOH, each)
SEQ ID NO: 20
SEQ ID NO: 30
SEQ ID NO: 21

Fig. 4.

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EUROPEAN SEARCH REPORT

Application Number
EP 20 18 5646

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The present search report has been drawn up for all claims

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Place of search	Date of completion of the search	Examiner
Munich	8 October 2020	Griesinger, Irina

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